



University
of Glasgow

<https://theses.gla.ac.uk/>

Theses Digitisation:

<https://www.gla.ac.uk/myglasgow/research/enlighten/theses/digitisation/>

This is a digitised version of the original print thesis.

Copyright and moral rights for this work are retained by the author

A copy can be downloaded for personal non-commercial research or study,
without prior permission or charge

This work cannot be reproduced or quoted extensively from without first
obtaining permission in writing from the author

The content must not be changed in any way or sold commercially in any
format or medium without the formal permission of the author

When referring to this work, full bibliographic details including the author,
title, awarding institution and date of the thesis must be given

Enlighten: Theses

<https://theses.gla.ac.uk/>
research-enlighten@glasgow.ac.uk

MODULATION OF DRUG RESISTANCE
IN SMALL CELL LUNG CANCER

by

Robert Milroy

M.B., Ch.B., M.R.C.P.

A Thesis Submitted for the Degree of

Doctor of Medicine

to

The University of Glasgow

**From Research Conducted in The CRC Department of
Medical Oncology, University of Glasgow and The
Department of Respiratory Medicine, Royal Infirmary,
Glasgow.**

June 1990

ProQuest Number: 11007567

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.



ProQuest 11007567

Published by ProQuest LLC (2018). Copyright of the Dissertation is held by the Author.

All rights reserved.

This work is protected against unauthorized copying under Title 17, United States Code
Microform Edition © ProQuest LLC.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

CONTENTS**Page**

Contents	2 - 10
List of Tables	11 - 14
List of Figures	15 - 17
List of Plates	18 - 19
Acknowledgements	20 - 22
Declaration	23 - 24
Dedication	25
Summary	26 - 29

CHAPTER 1 : GENERAL INTRODUCTION

Drug Resistance in Small Cell Lung Cancer -

The Problem in Perspective: 30 - 33

Theories of Drug Resistance in the Clinic: 33 - 36

Laboratory Models of Drug Resistance -

The Role of P-glycoprotein: 36 - 40

P-glycoprotein in the Clinic: 40 - 42

Modulation of P-glycoprotein Activity: 42 - 43

Verapamil: 43 - 45

Other Calcium Antagonists: 46

Quinidine: 47

Calmodulin Inhibitors: 47 - 48

Cyclosporins: 48 - 49

Amiodarone: 49 - 50

Aims of the Study: 50 - 51

Layout of the Thesis: 52 - 53

<u>CHAPTER 2 : ESTABLISHMENT OF SMALL CELL</u>	<u>Page</u>
<u>LUNG CANCER CELL LINES IN VITRO</u>	
<u>INTRODUCTION</u>	54 - 58
Establishment of SCLC Cell Lines In Vitro:	55 - 58
<u>METHODS</u>	59 - 64
Tumour Samples and Biopsy Techniques:	59 - 60
Media:	60 - 61
Disaggregation:	61 - 62
Collagenase:	62
STO Feeder Layers:	62
Conditioned Medium:	63
Maintaining Cells in Culture:	63
Specific Experiments:	63 - 64
<u>RESULTS</u>	65 - 72
Clinical History of Cell Lines:	66 - 71
Results of Specific Experiments:	72
<u>DISCUSSION</u>	73 - 78
Specific Experiments:	77 - 78
Summary:	78
 <u>CHAPTER 3 : CHARACTERISATION OF NEWLY</u>	
<u>ESTABLISHED SMALL CELL LUNG CANCER CELL LINES</u>	
<u>INTRODUCTION</u>	79 - 90
Classification of SCLC into Classic and	
Variant Sub-Groups:	79 - 80
The Heterogeneity of Lung Cancer:	80 - 83
Light Microscopy:	83 - 84

<u>INTRODUCTION (continued)</u>	<u>Page</u>
Electron Microscopy:	84 - 85
Immunocytochemistry:	85 - 87
Cytogenetic Characteristics:	87 - 88
Enzyme Activities:	89 - 90
<u>METHODS</u>	91 - 99
Cell Lines:	91
Light Microscopy:	91
Electron Microscopy:	91 - 92
Immunocytochemistry:	92 - 94
Cytogenetic Analyses:	94 - 95
Estimation of Dopa-Decarboxylase Activity:	95 - 97
Estimation of Creatine Kinase Activity:	97 - 99
<u>RESULTS</u>	100 - 103
Light Microscopy:	100
Electron Microscopy:	100 - 101
Immunocytochemistry:	101
Cytogenetic Analyses:	102
Measurement of Enzyme Activities:	102 - 103
<u>DISCUSSION</u>	104 - 110
Light Microscopy:	105
Electron Microscopy:	105 - 106
Immunocytochemistry:	106 - 107
Cytogenetic Analyses:	108 - 109
Biochemical Studies:	109 - 110
Summary:	110

<u>CHAPTER 4 : ESTIMATION OF GROWTH RATE</u>	<u>Page</u>
<u>AND CHEMOSENSITIVITY BY AGGREGATE GROWTH</u>	
<u>AND GROWTH DELAY ANALYSIS</u>	
<u>INTRODUCTION</u>	111 - 120
Clonogenic Assays:	112 - 114
Alternative Techniques for Measuring Growth and Chemosensitivity:	114
Proliferation Assays:	115 - 116
Metabolic Assays:	116 - 117
Assays of the Entire Cell Population:	117
Cell Membrane Function Assays:	117 - 119
Spheroid Growth Studies:	119 - 120
<u>METHODS</u>	121 - 122
Growth Studies:	121
Cytotoxicity Assays:	121 - 122
<u>RESULTS</u>	123 - 124
Growth Studies:	123 - 124
Cytotoxicity Assays:	124
<u>DISCUSSION</u>	125 - 127

CHAPTER 5 : ADAPTATION OF A TETRAZOLIUM
DYE-BASED ASSAY TO MEASURE CHEMOSENSITIVITY

<u>INTRODUCTION</u>	128 - 131
History of Tetrazolium Dyes in Science:	129 - 131
<u>METHODS</u>	132 - 138
Cell Lines:	132
Determination of Absorption Spectra:	133

<u>METHODS (continued)</u>	<u>Page</u>
Determination of Optimal MTT Concentration:	133 - 134
Determination of Optimal Incubation Time:	134
Relationship Between MTT-Formazan Production and Cell Number:	134 - 135
Growth Rates:	135
Chemosensitivity Measurements:	135 - 137
Effect of Time after Drug Exposure on the Apparent Chemosensitivity of the Cells:	137 - 138
Doxorubicin Penetration into and MTT-Formazan Production by Aggregates from Newly Established SCLC Cell Lines:	138
<u>RESULTS</u>	139 - 145
Spectral Analysis:	139
Determination of Optimal MTT Concentration:	139
Determination of Optimal Incubation Time:	140
Relationship between MTT-Formazan Production and Cell Number:	140
Effect of Cell Number on the Absorption Spectrum of MTT-Formazan:	140
Effect of pH on MTT-Formazan Absorbance:	141
Effect of Buffer Addition on the Relationship between MTT-Formazan Production and Cell Number:	141
Effect of Culture Medium on MTT-Formazan Production:	142
Growth Studies:	142

RESULTS (continued)**Page**

Effect of Buffer Addition on the Apparent

Chemosensitivity to Doxorubicin: 143

Effect of Recovery Time on Chemosensitivity: 143 - 144

Determination of Chemosensitivity by the

Microtitre Assay and Correlation with

a Clonogenic Assay: 144

Doxorubicin Penetration and MTT-Formazan

Production in Aggregates from Newly

Established SCLC Cell Lines: 145

DISCUSSION

146 - 160

pH Dependence of the MTT Assay: 149 - 152

Chemosensitivity Testing: 152 - 156

Solvents Used: 157 - 158

Limitations of the Assay: 158 - 159

Summary: 159 - 160

CHAPTER 6 : RESISTANCE MODULATION IN LUNG**CANCER CELL LINES****INTRODUCTION**

161 - 163

METHODS

164 - 167

Cell Lines: 164

Reagents/Drugs: 164 - 165

Determination of Optimal MTT Concentration: 165

Estimation of Growth Rates: 165 - 166

Cytotoxicity Assays: 166 - 167

	<u>Page</u>
<u>RESULTS</u>	168 - 172
Determination of Optimal MTT Concentration:	168
Growth Studies:	168
Determination of Chemosensitivities of	
Newly Established SCLC Cell Lines:	168 - 169
Effects of Racemic Verapamil and its	
Stereoisomers on Drug Sensitivity in H69LX10:	169
Concentration Dependent Effect of Verapamil:	170
Effect of Pre-treatment with Verapamil:	170
Effects of Other Resistance Modifiers in H69LX10:	171
Effects of Resistance Modifiers in Established	
NSCLC Cell Lines:	171
Effects of Verapamil in Newly Established	
SCLC Cell Lines:	172
<u>DISCUSSION</u>	173 - 179
Chemosensitivity of Newly Established SCLC	
Cell Lines:	173 - 176
Resistance Modulation in NSCLC and an MDR SCLC	
Cell Lines:	176 - 178
Resistance Modulation in Newly Established	
SCLC Cell Lines:	178 - 179
 <u>CHAPTER 7 : A RANDOMISED CLINICAL STUDY OF</u>	
<u>VERAPAMIL IN ADDITION TO COMBINATION</u>	
<u>CHEMOTHERAPY IN SMALL CELL LUNG CANCER</u>	
<u>INTRODUCTION</u>	180 - 185

INTRODUCTION (continued)

Previous Clinical Studies: 180 - 183

METHODS

Patient Selection: 186

Registration, Stratification, Randomisation
and Data Collection: 186 - 187

Pre-Treatment Studies: 187

Treatment Design: 187 - 188

Dose Modification: 188

Follow-up Studies and Restaging: 189

Verapamil Levels: 189

Confirmation of Response: 190

Statistical Methods: 190 - 191

RESULTS 192 - 196

Patient Demography: 192

Total Drug Doses and Number of Cycles
of Protocol Treatment: 192 - 193

Reasons for Discontinuing Chemotherapy: 193

Toxicity: 193 - 194

Response: 194 - 195

Confirmation of Response: 195

Survival: 195 - 196

Verapamil Levels: 196

DISCUSSION 197 - 204

Toxicity: 197 - 199

Response: 199 - 200

Survival: 200 - 201

DISCUSSION (continued)**Page**

Criticism of Study Design:

201 - 202

Summary:

202 - 204

CHAPTER 8 : GENERAL DISCUSSION

Establishment and Characterisation of SCLC

Cell Lines:

205 - 206

Chemosensitivity Testing:

206 - 209

Clinical Study of Verapamil Plus Chemotherapy
in SCLC:

209 - 210

Possible Mechanisms of Resistance in SCLC -

P-glycoprotein Reviewed:

211 - 213

Alternative Mechanisms of Resistance:

213

Glutathione and Glutathione-S-Transferases:

213 - 216

Topoisomerases:

216 - 217

Future Direction of Clinical Research:

217 - 218

P-glycoprotein Blockers:

218 - 219

GST Antagonists:

220

Concluding Comment:

220

REFERENCES

221 - 286

LIST OF TABLES**Following Page**

<u>Table 2.1</u>	Sources of Biopsies and Methodology for Establishing SCLC Cell Lines:	65
<u>Table 3.1</u>	Sources and Antigenic Determinants of Antibodies:	93
<u>Table 3.2</u>	Scale for Grading Intensity of Antibody Staining:	94
<u>Table 3.3</u>	Ultra-structural Features of the SCLC Cell Lines:	100
<u>Table 3.4</u>	Immunocytochemical Studies in SCLC Cell Lines:	101
<u>Table 3.5</u>	Cytogenetic Analyses of SCLC Cell Lines:	102
<u>Table 3.6</u>	Activities of the Enzymes DDC and CK in SCLC Cell Lines:	102
<u>Table 3.7</u>	Parameters Used to Classify SCLC Cell Lines:	104

Following Page

<u>Table 3.8</u>	Classification of Newly Established SCLC Cell Lines:	104
<u>Table 4.1</u>	Doubling Times and Growth Delays caused by Doxorubicin in SCLC Cell Lines Measured by Spheroid Image Analysis:	124
<u>Table 5.1</u>	Effect of Culture Medium on MTT-F Production:	142
<u>Table 5.2</u>	Effect of Recovery Time on Chemosensitivity:	143
<u>Table 5.3</u>	Correlation of Chemosensitivity Determined by Microtitre and Clonogenic Assays:	144
<u>Table 5.4</u>	Determination of Co-efficient of Variation for the MTT Assay:	144
<u>Table 6.1</u>	Doubling Times and Chemosensitivities of Newly Established SCLC Cell Lines:	168
<u>Table 6.2</u>	Effects of Racemic Verapamil and its Stereoisomers on Drug Sensitivity in H69LX10:	169

	<u>Following Page</u>
<u>Table 6.3</u> Effect of Pre-treatment with Verapamil:	170
<u>Table 6.4</u> Effects of Other Resistance Modifiers in H69LX10:	171
<u>Table 6.5</u> Effects of Resistance Modifiers in Established NSCLC Cell Lines:	171
<u>Table 6.6</u> Effects of Verapamil in Newly Established SCLC Cell Lines:	172
<u>Table 7.1</u> Details of Study Patients:	192
<u>Table 7.2</u> Details of Pre-treatment Patient Characteristics:	192
<u>Table 7.3</u> Total Drug Doses and Number of Cycles of Protocol Treatment:	192
<u>Table 7.4</u> Reasons for Discontinuing Chemotherapy and Causes of Death during Treatment:	193
<u>Table 7.5</u> General Toxicities in Study Patients:	193
<u>Table 7.6</u> Cardiovascular Parameters in Patients Treated with Verapamil:	194

Following Page

<u>Table 7.7</u>	Nadir Blood Count Values after Each Course of Chemotherapy:	194
<u>Table 7.8</u>	Worst Recorded Haematological Toxicity in Study Patients:	194
<u>Table 7.9</u>	Incidence of Chemotherapy Dose Reductions and Delays:	194
<u>Table 7.10</u>	Response Data at 12 weeks for All Patients:	194
<u>Table 7.11</u>	Re-evaluation of Radiological Response Assessment:	195
<u>Table 7.12</u>	Analysis of Survival Data and Causes of Death:	196

LIST OF FIGURES**Following Page**

<u>Figure 4.1</u>	Growth Curves for Cell Lines LS111 and LS112FL Estimated by Spheroid Image Analysis:	123
<u>Figure 4.2</u>	Growth Curves for Cell Lines LS106 and LCPH3 Estimated by Spheroid Image Analysis:	123
<u>Figure 4.3</u>	Effects of Doxorubicin on Growth Patterns in Cell Lines LS111 and LS112FL Assessed by Spheroid Image Analysis:	124
<u>Figure 5.1</u>	Molecular Structure of MTT and the Reduced Formazan Product:	129
<u>Figure 5.2</u>	Absorption Spectra of MTT and MTT-F:	139
<u>Figure 5.3</u>	Determination of Optimal MTT Concentration:	139
<u>Figure 5.4</u>	Determination of Optimal Incubation Time for MTT:	140
<u>Figure 5.5</u>	Relationship between MTT-F Production and Cell Number:	140

Following Page

<u>Figure 5.6</u>	Effect of Cell Number on the Absorption Spectrum of MTT-F:	140
<u>Figure 5.7</u>	Effect of pH on MTT-F Absorbance:	141
<u>Figure 5.8</u>	Effect of Buffer Addition on the Relationship between MTT-F Production and Cell Number:	141
<u>Figure 5.9</u>	Effect of HEPES Buffer Addition During MTT Incubation:	141
<u>Figure 5.10</u>	Growth in L-DAN and GLC4 Studied using MTT Assay:	142
<u>Figure 5.11</u>	Effect of Buffer on Chemosensitivity Measured by MTT Assay:	143
<u>Figure 5.12</u>	Effect of Recovery Time on Chemosensitivity:	143
<u>Figure 5.13</u>	Determination of Chemosensitivity of L-DAN and GLC4 to Doxorubicin using MTT Assay:	144

Following Page

<u>Figure 6.1</u>	Determination of Optimal MTT Concentration in SCLC Cell Lines:	168
<u>Figure 6.2</u>	Growth Curves for Newly Established SCLC Cell Lines:	168
<u>Figure 6.3</u>	Determination of Sensitivity to Doxorubicin of LCPH2 using the MTT Assay:	168
<u>Figure 6.4</u>	Determination of Sensitivity to Doxorubicin of LS111 using the MTT Assay:	169
<u>Figure 6.5</u>	Concentration Dependent Effect of Verapamil in H69LX10:	170
<u>Figure 6.6</u>	Effect of Verapamil on Sensitivity of LS263 to Doxorubicin Determined by the MTT Assay:	172
<u>Figure 7.1</u>	Survival Curves for Patients in the Randomised Clinical Study:	195

LIST OF PLATES**Following Page**

<u>Plate 1.1</u>	The Incidence of Lung Cancer in Scotland:	30
<u>Plate 1.2</u>	Series of Chest Radiographs Illustrating an SCLC Tumour which Initially Responds Completely to Chemotherapy but then Recurs:	31
<u>Plate 1.3</u>	The Structure of P-glycoprotein:	38
<u>Plate 2.1</u>	Viable Clumps of Tumour Cells at an Early Stage in Tissue Culture:	65
<u>Plate 2.2</u>	Aggregate from Cell Line LS111:	66
<u>Plate 2.3</u>	Two Cell Populations Derived from Biopsy LS112:	66
<u>Plate 3.1</u>	Section of LS111 Tumour Cell Aggregate (Stained with Haematoxylin and Eosin):	100
<u>Plate 3.2</u>	Transmission Electron Micrograph Prepared from Cell Line LS111:	100

Following Page

<u>Plate 3.3</u>	Positive Staining with the Antibody	
	CAM 5.2 in Cell Line LS263:	101
<u>Plate 3.4</u>	Karyotype of a Representative Metaphase	
	of Cell Line LS111:	102
<u>Plate 4.1</u>	Similarities between Tumour Aggregates	
	In Vitro and Tumour In Vivo:	112
<u>Plate 4.2</u>	The Image Analysis Apparatus Used for	
	Spheroid Growth Studies:	121
<u>Plate 4.3</u>	The Non-Spheroidal Appearance of an	
	Aggregate from Cell Line LS112FL:	126
<u>Plate 4.4</u>	Damaged Aggregate from Cell Line LS111:	126
<u>Plate 5.1</u>	Distribution of Doxorubicin and MTT-F	
	in Aggregates from Cell Line LS111:	145
<u>Plate 5.2</u>	Distribution of Doxorubicin and MTT-F	
	in Aggregates from Cell Line LCPH3:	145
<u>Plate 6.1</u>	Microtitre Plate after Completion of	
	MTT Assay to Measure Chemosensitivity	
	of SCLC Aggregates:	168

ACKNOWLEDGEMENTS

I owe most grateful thanks for help, encouragement, advice and support to my supervisor:

Dr. Jane A. Plumb,

CRC Research Fellow in Oncology, University of Glasgow.

and also to:

Professor S.B. Kaye,

CRC Professor of Medical Oncology, University of Glasgow.

and

Dr. S.W. Banham,

Consultant Physician in Respiratory Medicine, Royal Infirmary, Glasgow.

I am most grateful to the Cancer Research Campaign for providing a two year grant for a Research Fellowship which enabled me to research this thesis.

Thanks are also due to:

Dr. Ruth Adamson

Senior Registrar, Department of Pathology, Royal Infirmary, Glasgow.

Mr. James Anderson

Scientist, Department of Pathology, Royal Infirmary, Glasgow.

Mr. Paul Batstone	Scientist, Cytogenetics Unit, Department of Pathology, Ninewells Hospital, Dundee.
Dr. Steven Bicknell	CRC Research Fellow, Department of Medical Oncology, University of Glasgow.
Dr. William Candlish	Senior Registrar, Department of Pathology, Royal Infirmary, Glasgow.
Ms. Linda Cramm	Data Manageress, Beatson Oncology Centre, Western Infirmary, Glasgow.
Ms. Moira Farquharson	Scientist, Department of Pathology, Royal Infirmary, Glasgow.
Mr. Philip Harris	Photographer, Department of Medical Illustration, Royal Infirmary, Glasgow.
Dr. Frances Hay	Scientist, ICRF Department of Medical Oncology, Western General Hospital, Edinburgh.

Ms. Diane Lindsay	Librarian, Medical Library, Royal Infirmary, Glasgow.
Dr. Arthur Maclay	Consultant Pathologist, Department of Pathology, Royal Infirmary, Glasgow.
Mrs. Elizabeth McLean	Secretary, Chest Clinic, Belvidere Hospital, Glasgow.
Mr. James Paul	Statistician, Beatson Oncology Centre, Western Infirmary, Glasgow.
Dr. Thomas Wheldon	Radiobiologist, Department of Radiobiology, Belvidere Hospital, Glasgow.

And also to:

The clinicians who entered patients into the randomised study.

Finally, special thanks are due to Mrs Elaine Riley, who typed this manuscript.

DECLARATION

I declare that the work presented in this thesis has been carried out solely by me, except where indicated in the text and below.

The characterisation of the cell lines was performed in collaboration with the following: light microscopy, Dr. R. Adamson and Dr. W. Candlish; electron microscopy, Dr. A. Maclay and Mr. J. Anderson; immunocytochemical studies, Ms. M. Farquharson, Dr. W. Candlish and Dr. R. Adamson, all from the Department of Pathology, Royal Infirmary, Glasgow. Additional immunocytochemical characterisation was undertaken jointly with Dr. Frances Hay, ICRF Department of Medical Oncology, Western General Hospital, Edinburgh. The cytogenetic studies were performed in collaboration with Mr. Paul Batstone, from the Cytogenetics Unit, Department of Pathology, Ninewells Hospital, Dundee. This work was presented at the British Association for Cancer Research Meeting in Norwich (April, 1988).

The adaptation of the tetrazolium dye-based assay was performed jointly with my supervisor, Dr. Jane A. Plumb. This work was presented at the American Thoracic Society Meeting in Las Vegas (May, 1988). The studies of resistance modulation in lung cancer cell lines have been presented at the British Thoracic Society Meeting in London (December, 1988) and at the American Association for Cancer Research Meeting in San Francisco (May, 1989).

The results of the randomised clinical study were coordinated by myself on behalf of the West of Scotland Lung Cancer Research Group in collaboration with Mr. James Paul and Ms. Linda Cramm, from the Beatson Oncology Centre, Western Infirmary, Glasgow. The interim results of this work were presented at the Fifth World Conference on Lung Cancer in Interlaken (August, 1988).

DEDICATION

To my mother and the memory of my late father.

SUMMARY

The aim of these studies was to modulate drug resistance in small cell lung cancer (SCLC) both in the laboratory and in the clinic with verapamil. Nine small cell lung cancer cell lines have been established from both pretreatment and relapse biopsies and include a pair of cell lines from the same patient. Problems in establishing cell lines related to lack of viable tumour material and overgrowth by fibroblasts.

All the cell lines have been characterised and demonstrate features characteristic of SCLC in vitro. All cell lines expressed the small cell marker enzymes dopa-decarboxylase and the BB isoenzyme of creatine kinase though the levels varied between cell lines. Pathological, immunocytochemical and ultrastructural studies were used to identify both epithelial and neuroendocrine features. Thus the cell lines could be classified into classic or variant phenotypes. Detailed cytogenetic analysis of the cell lines confirmed their human origin and all were shown to contain the 3p deletion characteristic of lung tumours. The modal chromosome number ranged from 39 to 53.

The cell lines all grow as non-adherent aggregates except for a monolayer variant of LS112. Measurement of growth rates and chemosensitivity therefore proved difficult since the aggregates could not be dispersed into a viable single cell suspension. Initially measurements were attempted by computerised spheroid image analysis. This technique was slow but provided limited

information for three of the cell lines (LS106, LS111 and LS112FL). However, the other cell lines tested did not grow well in this system.

A tetrazolium dye-based microtitration assay was therefore modified for use with both adherent and non-adherent cell lines. The assay uses as an end point the ability of live, but not dead cells, to reduce a yellow water-soluble tetrazolium dye (MTT) to a purple water-insoluble formazan product (MTT-formazan). Initial work with this assay indicated a number of deficiencies and, in particular, at high cell density or low pH the relationship between MTT-formazan production and cell number was not linear. It was shown that by determining the optimal concentration of MTT for each cell line and by addition of a buffer at a high pH (10.5) to control the pH of the formazan product a linear relationship could be obtained. When intact aggregates were incubated with MTT, crystals of MTT-formazan were produced by the entire viable cell population of the aggregate. Furthermore, doxorubicin was shown to penetrate throughout the viable cell population of the aggregate. Thus the assay could be applied to the intact SCLC aggregates thereby avoiding the need to disrupt the clusters with the consequent loss of cell interaction and viability.

The cell lines were slow growing with doubling times of between 4 and 6 days. There was a 60-fold range in sensitivity to doxorubicin (ID_{50} , 17.5 - 1050nM). Intrinsic sensitivity to doxorubicin did not relate either to any specific characteristic of the cell lines or to the history of the patient from whose biopsy the cell line was established. However, for the pair of cell lines

established from the same patient the line derived from the relapse biopsy (LS310) was five-fold more resistant to doxorubicin than the line derived from the original chemosensitive pretreatment biopsy (LS274).

For the multidrug resistant SCLC cell line, H69LX10, resistance modification by verapamil was shown to be dose dependent and was maximal (10-fold) at about 6-7 μM . At clinically achievable levels of verapamil (1 μM) the effect was only 2-fold. These results indicate that verapamil may have limited activity in the clinic. However, the D-stereoisomer of verapamil was shown to be an equally effective resistance modulator as the racemic mixture. Since D-verapamil is thought to be less cardiotoxic than L-verapamil it is possible that use of D-verapamil in the clinic will allow increased plasma levels of the modulator without undue cardiotoxicity. Quinidine and bepridil were also identified as modulators that have significant activity in H69LX10 cells in the laboratory at levels achievable in the clinic.

An increase in sensitivity to doxorubicin (2-3 fold) in the presence of verapamil (6.6 μM) was demonstrated in 5 of the 9 newly established SCLC cell lines. The sensitising effect of verapamil did not relate to patient history or to the intrinsic sensitivity of the cell line to doxorubicin. Thus, the most sensitive line (LS277) established from an untreated patient showed a 2.3-fold increase in sensitivity whereas the most resistant line (LS310) from a relapse biopsy showed no change in sensitivity when co-incubated with verapamil.

In order to assess whether verapamil might have an effect on

relapse with resistant tumour in the clinic the first randomised large-scale (comprising 226 patients) study of verapamil given in addition to combination chemotherapy in patients with SCLC was undertaken. All patients received four cycles of identical combination chemotherapy (including doxorubicin) with or without verapamil. Toxicity in both arms of the study was similar. In particular, there was no undue cardiovascular toxicity. There were no major differences in response rate or in survival between the control and verapamil arms.

Thus the role of verapamil as a resistance modifier in the clinical management of small cell lung cancer remains unclear. Problems may relate to the relatively low levels of verapamil achievable in the clinic. Furthermore, the importance of P-glycoprotein expression as a resistance mechanism in SCLC is not clear. If over-expression of P-glycoprotein is not a common mechanism of drug resistance in SCLC, future attempts to modulate drug resistance should be aimed at other resistance mechanisms such as altered activities of drug detoxification enzymes or of topoisomerases.

Nonetheless, this work demonstrates for the first time clear evidence of resistance modification by verapamil in newly established SCLC cell lines and identifies two modulators, quinidine and bepridil, that are active at levels achievable in the clinic.

CHAPTER I

GENERAL INTRODUCTION

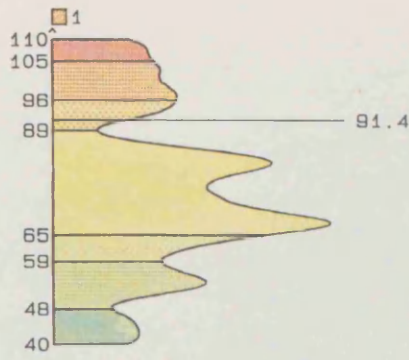
Drug Resistance in Small Cell Lung Cancer:

The Problem in Perspective

In this country, lung cancer is the commonest malignancy in men and second most common malignancy in women. Although the incidence may now be reaching a plateau, lung cancer still causes 2,500 deaths every year in the West of Scotland (Kemp et al., 1985). Indeed the West of Scotland has the highest incidence of lung cancer in the Western world (Plate 1.1).

Small cell lung cancer (SCLC), represents about 25% of all lung tumours and is regarded as a distinct pathological entity. It differs from the other histological types of lung tumour with respect to its biological behaviour and its usually initial, dramatic response to cytotoxic agents (Seifter & Ihde, 1988).

The natural history of SCLC is characterised by a progressive clinical course with the early development of metastases (Cohen & Mathews, 1978; Morstyn et al., 1984). Indeed in comparison with other types of lung cancer, patients presenting with SCLC often have a shorter history of symptoms, the majority have overt evidence of widespread tumour dissemination at presentation and in the absence of therapy their disease progresses relentlessly. Thus SCLC has come to be regarded as a systemic disease at presentation.



D=13.00, $p<0.0001$

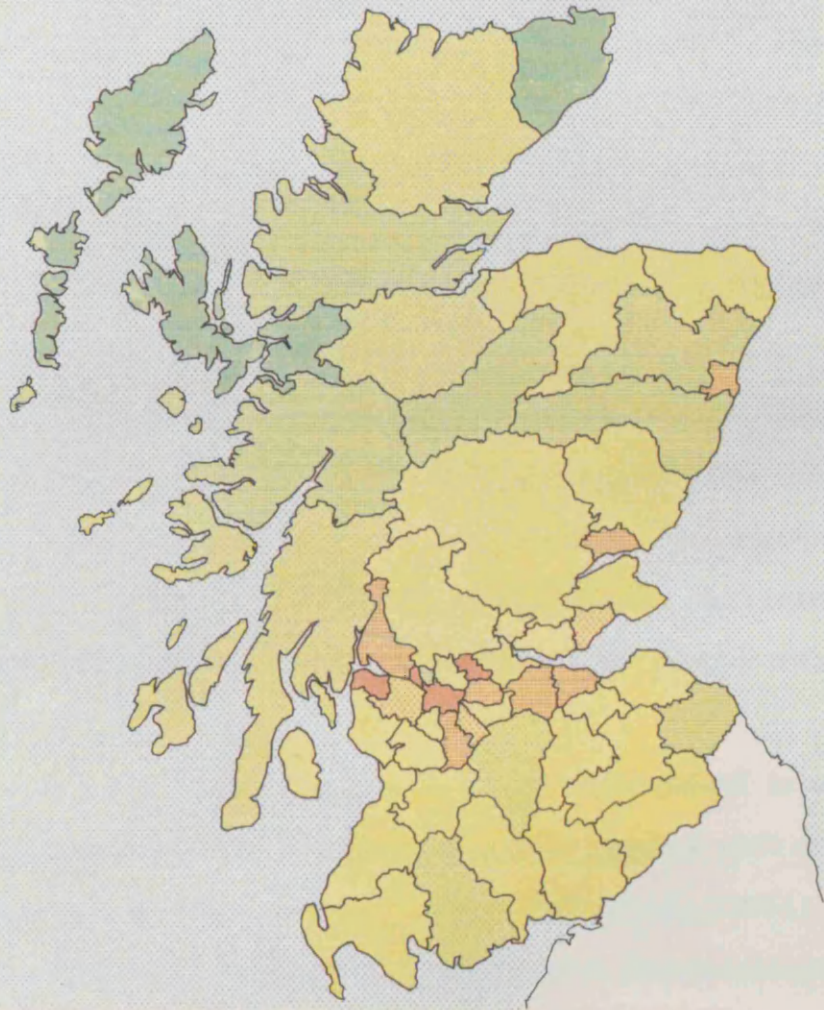


Plate 1.1

Map of Scotland illustrating the annual average incidence of lung cancer per 100,000 population (1975-1980) (from: Kemp et al., 1985).

Over the last two decades it has become clear that SCLC is usually responsive to a wide range of cytotoxic drugs. This has led to the establishment of chemotherapy as the mainstay of treatment for this disease (Aisner et al., 1983). Aggressive therapeutic regimes yield the best response rates and generally produce the highest proportion of long-term survivors in SCLC (Aisner et al., 1983). However despite response rates of more than 80% and significantly improved median survival (10-12 months) with combination chemotherapy, at best about 10% of patients will remain disease-free 2 years after diagnosis (Ihde, 1984; Morstyn et al., 1984). The reason for such disappointing long-term survival statistics relates to relapse with tumour which is generally resistant to most cytotoxic agents (Aisner et al., 1983).

Thus relapse with chemoresistant tumour is the main cause for treatment failure in SCLC. Plate 1.2 exemplifies this problem. This series of chest radiographs show the original tumour (A), a dramatic response to chemotherapy (B), and then after a prolonged period of remission the untreatable relapse with resistant tumour (C).

This is not a feature unique to SCLC, and indeed is a problem in many other commonly occurring solid tumours such as breast (Benson 111 et al., 1985) and ovary (Ozols et al., 1987A). Indeed the frustrating phenomenon of relapse with chemoresistant tumour has been evident since cytotoxic agents capable of inducing effective tumour regression were first used (Farber et al., 1948).

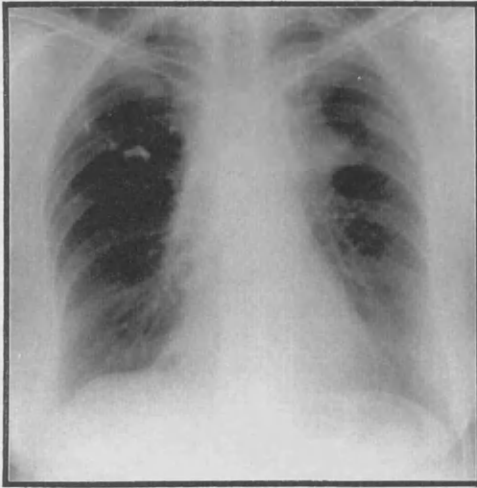
The nature of resistance in such tumours remains unclear. It is probable that a range of mechanisms of resistance operate.



A



B



C

Plate 1.2

Chest radiograph of middle-aged female with small cell lung cancer at presentation showing extensive left sided tumour (A). There was a complete radiological response (confirmed by re-staging bronchoscopy) to induction chemotherapy (B). Twenty-four months later the patient relapsed locally (C). This relapse tumour proved resistant to further chemotherapy.

It may be that relapse with resistant tumour is a manifestation of resistance that has developed secondary to cytotoxic therapy (so-called acquired resistance). Luria & Delbruck (1943) first described acquired resistance due to selection of spontaneously occurring mutations in bacteriophage. Similar spontaneous mutations might explain resistance to cytotoxic drugs in mammalian cancer cells (Isaacs & Coffey, 1981; Law, 1952). More recently a mathematical model for the process of spontaneous mutation resulting in acquired drug resistance has been developed by Goldie & Coldman (1979). Alternatively, such relapse might relate to the overgrowth of a population of intrinsically resistant tumour cells, present from the start in the natural history of the tumour (i.e. intrinsic resistance).

Numerous studies have shown that there are sub-populations of cells in animal tumours (Barranco et al., 1978; Lotan & Nicolson, 1979) and human tumours (Barranco et al., 1972; Trope et al., 1975) with differing susceptibilities to a variety of cytotoxic agents. More recently Tsuruo & Fidler (1981) demonstrated extensive differences in drug sensitivity among cells populating parental tumour and metastatic sub-populations. Thus metastases may result from the growth of selected sub-populations of cells that pre-exist within the primary neoplasm (Fidler & Kripke, 1977; Poste & Fidler, 1980). The recognition that neoplasms are populated by cells with such diverse biological behaviour has prompted investigation into both the nature of the biological heterogeneity of neoplasms and also into the implications of these findings for therapy (Calabresi et al., 1979; Fidler, 1978; Fidler

& Kripke, 1977; Kerbel, 1979). Indeed micrometastases and metastases have been shown to be genetically unstable and so more likely to become resistant (Fidler, 1978).

Small cell lung cancer, by its very biological nature, with its clinical behaviour comprising a brisk initial response to chemotherapy followed by relapse with resistant tumour, serves as an ideal model for the study of drug resistance both in the laboratory and in the clinic. The study of resistance in any tumour model system comprises both the investigation of possible mechanisms of the resistance, and also the application of methods to try and overcome such resistance. A great deal of our current understanding of mechanisms of resistance and methods of circumventing resistance has come from laboratory models of drug resistant tumour cell lines. Before considering what information has been obtained from such in vitro models of resistance and of what clinical relevance these findings might be, it is first appropriate to review the general history of concepts of resistance in the clinical setting.

Theories of Drug Resistance in the Clinic

There are a number of possible components of tumour resistance to cytotoxic drugs in the clinic. The patient might handle drugs in an altered fashion in terms of drug absorption and activation. Altered tumour vascularisation could result in reduced penetration of drug into the tumour. However, most theories concerning tumour resistance are based on cellular mechanisms.

This might relate to any or all of the following factors: the proliferative state of the cell, altered cellular biochemistry, and altered cellular pharmacology (drug uptake and retention). Before considering each of these three broad groupings it is worth noting that many cytotoxic drugs themselves are potent mutagens and may themselves directly induce resistant mutants (Selby, 1984). Furthermore tumour cells may interact (both in vivo and in vitro) and these interactions may influence drug sensitivity (Miller et al., 1981).

Initially the popular belief was that the main mechanism of resistance related to altered cell kinetics. The most resistant cell was thought to be the non-proliferating (Go) cell. The frequency of resistance in solid tumours was thought to relate to the large proportion of Go cells and consequent low growth fraction and long doubling time in these tumours. This theory has been elegantly reviewed by Carter (1984). However, there is little evidence to suggest that initially sensitive cells may become resistant by altered proliferation kinetics.

Subsequently, a biochemical view became popularised. This led to the development of anti-metabolites and between 1950 and 1970 a massive data base of "rationally designed anti-metabolites" was developed. In terms of tumour cell resistance, it seems likely that altered biochemistry plays a major role (see below).

More recently a pharmacological view has been developed to explain the lack of activity of cytotoxic drugs in resistant tumour cells. This was based on the premise that a cytotoxic drug could only kill tumour cells if it reached the cell at an adequate

concentration and for an adequate time. This led to a great number of studies of the pharmacokinetics of cytotoxic agents but these only developed some post-hoc rationalisation for why one empirically discovered schedule was superior to others (Carter, 1984). Nonetheless, in terms of resistant tumour, it seems likely that altered drug handling does play a role.

By the 1980s the view of cancer chemotherapy resistance was an integration of the proliferative, biochemical and pharmacological concepts, all placed within the broad conceptual context of the cell kill hypothesis (Carter, 1984). The cell kill hypothesis was first elucidated by Skipper and Schabel (1984). This stated that if the dose of cytotoxic drug could be increased to a sufficiently high level in relation to the total number of tumour cells present, then the fractional kill would lead to the total eradication of the tumour mass and hence lead to cure. However, this approach did not lead to cure. The paradigm to explain this failure of chemotherapy focuses on the problem of tumour cell resistance.

Whether this is inherent or acquired during chemotherapy, a model of tumour cell resistance predicts that there will be a proportion of resistant cells within a tumour. The final common pathway of all chemotherapy strategies is to overcome such resistant tumour cells, and completely eradicate them at cytotoxic dose levels that are tolerated by the host. Such a model of tumour cell resistance supports the administration of the highest possible dose of cytotoxic drug but does recognise that if resistance has developed, then the tumour will not be successfully eradicated simply by dose escalation. This model remains an essentially

proliferative model but shifts the emphasis from tumour cell kinetics to tumour cell resistance. However, this conceptual model does not consider the mechanism of tumour cell resistance or ways of overcoming it, although it naturally leads to such concepts. Nor does this model consider the possibility that resistance may relate to tumour cell dormancy. Such mitotic quiescence may relate to a lack of or inactivation of essential tumour growth factors. Alternatively some tumour cells may undergo differentiation associated with reversible cessation of division (Alexander, 1982; Carter, 1984). Tumour cell dormancy remains a potentially formidable problem. Nonetheless, from clinical experience it seems likely that the failure of chemotherapy to cure the vast majority of patients with SCLC relates to the overgrowth of resistant tumour cells.

Laboratory Models of Drug Resistance: The Role of P-glycoprotein

A number of laboratory models of drug resistance have now been developed. Tumour cell lines can be made resistant by chronic exposure to increasing doses of cytotoxic agents. This approach, which results in cell lines with a high degree of acquired resistance was followed by a number of workers (Akiyama et al., 1985A; Beck et al., 1979; Biedler & Riehm, 1970; Ling & Thompson, 1974; Shen et al., 1986A). Such cell lines made resistant to one cytotoxic agent often demonstrate cross-resistance to a broad range of structurally unrelated cytotoxic drugs - so called multi-drug resistance (MDR). Although initial studies

involved resistance to actinomycin D (Biedler & Riehm, 1970) and vinca alkaloids and colchicine (Ling & Thompson, 1974), other cytotoxic agents have also been used, including etoposide (Seeber et al., 1982) and mitomycin-C (Dorr et al., 1987). These cells appear to be resistant because they have decreased intracellular cytotoxic accumulation, probably related to increased drug efflux (Fojo et al., 1985; Inaba & Johnson, 1978; Inaba et al., 1979; Nishimura et al., 1979).

More recent work examining genetic changes in resistant cells has demonstrated that these multi-drug resistant cells usually contain a gene, termed the MDR-1 gene, which is transcribed into a 4.5 kilobase messenger RNA (Gros et al., 1986A; Riordan et al., 1985; Scotto et al., 1986; Shen et al., 1986B; Van der Bliek et al., 1986). This gene is over-expressed but not always amplified in MDR cells (Fairchild et al., 1987; Roninson et al., 1984). The protein product of this gene is a 170 kilodalton membrane glycoprotein, named P-glycoprotein (P for permeability) which is thought to act as an energy dependent drug efflux pump (Gottesman & Pastan, 1988; Horio et al., 1988). This is now the most clearly understood mechanism of drug resistance in vitro and for this reason it is the only mechanism addressed by the studies described in this thesis.

A full length complementary DNA for the MDR-1 gene from one of the multi-drug resistant human KB carcinoma cell lines has been isolated and sequenced (Chen et al., 1986; Roninson et al., 1986; Ueda et al., 1987A,B). Molecular biological techniques were subsequently used to further study the function and possible

importance of the MDR-1 gene. Although Roninson's and Gros's work initially involved resistant animal tumour cells, Pastan and Gottesman focused on resistant human tumour mutants. The gene sequences in these human tumour mutants were remarkably similar to the DNA isolated by Gros from resistant hamster tumour (Chen et al., 1986; Gros et al., 1986A). The structure, amino-acid sequence and configuration of P-glycoprotein within the cell membrane have now been elucidated (Plate 1.3) and its remarkable resemblance to bacterial transport proteins noted (Gerlach et al., 1986; Gros et al., 1986B). This suggests it may have a role as a drug transport protein.

There is now evidence that P-glycoprotein is a member of a multigene family. At least two different classes of P-glycoprotein cDNA have been identified in hamster (Endicott et al., 1987), mouse (Gros et al., 1986A) and human (Van der Bliek et al., 1987) tumours. Certainly amplification of other closely juxtaposed gene classes has also been detected in MDR cells, the product of one of which is the cytoplasmic protein, sorcin (Jongsma et al., 1987).

The most dramatic evidence to suggest P-glycoprotein is important in multi-drug resistance arises from the experimental transfer of genetic material encoding for P-glycoprotein. First experiments involved transferring genomic DNA from MDR hamster cells into drug-sensitive mouse cells and with the use of species-specific anti-P-glycoprotein antibodies demonstrating the presence of hamster P-glycoprotein in the originally sensitive mouse tumour cells (Deuchars et al., 1987). Resistance was also shown to be conferred on mouse N.I.H. 3T3 cells by transfecting genomic DNA



Plate 1.3

Schematic diagram of the P-glycoprotein molecule. This illustration emphasises the trans-membrane location of this complex structure, which is thought to act as an energy-dependent drug efflux pump in MDR tumour cells. The ATP binding sites (green) are intracellular, whereas the carbohydrate groups (purple) lie on the cell surface. (from: Kartner & Ling, 1989).

from MDR human KB cells (Shen et al., 1986C). Furthermore, full length cDNAs coding for human P-glycoprotein have been sub-cloned into expression vectors and transfected into drug sensitive cells (Pastan et al., 1988; Ueda et al., 1987A,B). Thus it seems likely that P-glycoprotein can be implicated in MDR, although it may not be the only component of this phenomenon. Indeed there is a body of evidence against P-glycoprotein being the only mechanism of resistance in MDR cells.

Although MDR cell lines do in general express similar patterns of resistance, each individual cell line displays its own pattern of cross-resistance, which may be different from patterns of resistance in other MDR cell lines (Beck et al., 1979; Fairchild et al., 1987; Riordan et al., 1985; Roninson et al., 1984; Scotto et al., 1986). The number of different P-glycoprotein genes, even taking into account variation in levels of expression, seems insufficient to account for this diversity (Moscow & Cowan, 1988). Furthermore the degree of resistance of many cell lines to anti-cancer drugs is not directly correlated with the degree of intracellular accumulation of drug (Chang & Gregory, 1985; Louie et al., 1986; Siegfried et al., 1983; Sirotnak et al., 1986).

In addition some MDR cells display additional changes in protein expression. For example, sorcin is over-expressed and amplified in a vincristine resistant cell line, and this suggested that this protein might contribute to MDR. However, subsequent investigations indicated that the sorcin gene is simply a passenger gene (De Bruijn et al., 1986; Deuchars et al., 1987). Finally several human cell lines have recently been described that have

transport-mediated defects and a multi-drug resistant phenotype, but do not over-express P-glycoprotein (Dalton et al., 1988; Danks et al., 1987; Marsh & Center, 1987; Mirski et al., 1987; Slovak et al., 1988).

P-glycoprotein in the Clinic

Following the identification of P-glycoprotein in MDR resistant cells in vitro a number of studies have investigated the possible role of this protein in clinical drug resistance. These studies have been made possible by the development of monoclonal antibodies to P-glycoprotein (Bell et al., 1985, Sugawara et al., 1988) and by the isolation of a cDNA probe that can detect MDR-1 mRNA (Fojo et al., 1987B).

Fojo et al. (1987B) have shown that the MDR-1 gene is expressed at a high level in normal human kidneys, adrenal gland, liver and colon. Moreover Thiebaut et al. (1987) have shown that in kidney, liver and colon, the P-glycoprotein is present on the luminal surface of epithelial cells compatible with its role as a transporter.

Relatively high levels of MDR-1 mRNA have been found in normal colon and colon cancer and adjacent "normal" colon (Fojo et al., 1987B). In 2 of 8 patients higher levels of MDR-1 mRNA were seen in the tumour and the authors speculate that this could be related to the known intrinsic resistance of colonic carcinoma. In addition 6-fold higher levels of MDR-1 mRNA were detected in a relapse tumour than in the pre-chemotherapy biopsy. Similarly

Fojo et al. (1987A) have suggested that the intrinsic resistance seen in renal cancer is associated with expression of the MDR-1 gene.

Bell et al. (1985), using immunocytochemical techniques revealed over-expression of P-glycoprotein in 2 of 5 patients with ovarian carcinoma. Similar techniques have demonstrated P-glycoprotein over-expression in 2 patients with acute non-lymphocytic leukaemia (Ma et al., 1987). In 25 patients with sarcoma, Gerlach et al. (1987) demonstrated increased levels of P-glycoprotein in 6 tumour samples. More recently Goldstein et al., (1989) measured MDR-1 mRNA levels using a slot blot technique in an extensive range of tumour samples (human biopsy material and cell lines). They demonstrated a wide-range of MDR-1 mRNA levels, ranging from high (e.g. in colon, renal and liver cancer) to low/undetectable in breast, ovary, upper gastro-intestinal, small cell and non-small cell lung cancer (the latter was studied only in cell lines). They did report high levels of MDR-1 mRNA in relapse tumours, but, apart from breast, the tumours also had high pre-therapy levels. The authors go on to postulate that high MDR-1 mRNA levels are associated with tumours resistant in the clinic.

There have been few studies of P-glycoprotein expression in lung tumours. Recently Lai et al. (1989) reported the presence of MDR-1 mRNA in both SCLC tumour biopsies and in cell lines derived from SCLC. Although the levels were low when compared with other tumour types the significance of the level of expression is not known. Since the technique used did not consider

individual cells, the low levels found may represent a small focus of resistant tumour cells within a population of drug-sensitive cells.

It is clear that P-glycoprotein is an important component in the mechanism of MDR. It may also play a role in clinical resistance, however the plethora of conflicting reports concerning the presence or absence of P-glycoprotein, or variable levels of MDR-1 mRNA in both clinical samples and cell-lines indicates that the role of P-glycoprotein in clinical drug resistance remains unclear.

Modulation of P-Glycoprotein Activity

It has been shown in animal and human tumour models of multi-drug resistance, that a number of agents are capable of restoring sensitivity to a range of cytotoxic drugs in MDR tumour cells. Such agents (termed resistance modifiers) come from various groups of drugs which by themselves are not cytotoxic. The most potent modifiers appear to share a common molecular structure: they are typically hydrophobic molecules with two planar aromatic rings and a tertiary basic nitrogen atom with a positive charge at physiologic pH (Rothenberg & Ling, 1989).

In the last decade there has been growing interest in such non-chemotherapeutic agents which might enhance the cytotoxicity of conventional chemotherapy drugs (such as doxorubicin). A number of calcium channel blocking drugs (typified by verapamil and nifedipine) and calmodulin inhibitors (such as fluphenazine and

trifluoperazine) have been shown to be effective in enhancing the cytotoxicity of natural product anti-cancer drugs, primarily vinca alkaloids and anthracyclines. The greatest effects of such resistance modifying agents have been seen in MDR tumour cell models, which often express increased levels of P-glycoprotein (Beck et al., 1986; Harker et al., 1986; Skovsgaard et al., 1984; Tsuruo et al., 1984).

Verapamil

The earliest work describing an increase in sensitivity of resistant cells in the presence of a resistance modifying agent was that reported by Tsuruo et al. (1981) using verapamil. Not only did they report the phenomenon, but they also demonstrated that the increase in sensitivity related to increased intracellular accumulation of cytotoxic drug (vincristine and doxorubicin) in the resistant cells in the presence of modifier (Tsuruo et al., 1981, 1982). Resistant cells had previously been shown to have decreased intracellular accumulation of doxorubicin, primarily because of enhanced active efflux (Inaba & Johnson, 1978; Inaba et al., 1979) whereas doxorubicin entry into cells probably relates to passive Fickian diffusion (Dalmark & Storm, 1981).

Interestingly some increase in intracellular drug accumulation in the presence of modifier was also noted in the sensitive tumour cells (Tsuruo et al., 1982). However the sensitising effect of verapamil with vincristine and doxorubicin was much more marked in the drug resistant model, a resistant P388 leukaemia cell line

(40-fold and 10-fold respectively) than in the standard P388 cell line (4-fold and 2-fold respectively). This reversal of drug resistance with verapamil was subsequently confirmed by others (Beck et al., 1986; Slater et al., 1982).

Rogan et al. (1984) demonstrated an increase in sensitivity of human ovarian cancer cells to doxorubicin in the presence of verapamil. This effect was most clearly seen in resistant cell lines, but some sensitisation with verapamil was also noted in the parental cell lines. Although there was a marked increase in sensitivity with verapamil, with a clear dose-response effect noted, in highly resistant in vitro models verapamil did not fully restore sensitivity suggesting that other mechanisms of resistance may also be operating.

Although the sensitising effect of verapamil has been clearly established, the mechanism(s) whereby verapamil exerts this effect remain unclear. In this regard it should be noted that MDR cells have neither inhibitable voltage dependent calcium channels (Kessel & Wilberding, 1985; Ramu et al., 1984), nor differences in calmodulin levels (Tsuruo et al., 1984). Thus the mechanism(s) whereby verapamil enhances chemosensitivity must relate to other factors.

In the case of verapamil and other calcium-channel blockers, inhibition of voltage-gated potassium channels (De Coursey et al., 1985) and certain calmodulin-dependent activities (Epstein et al., 1982; Janis & Scriabine, 1983) may be relevant. More recently it has been shown that verapamil can compete with labelled vinblastine for binding to isolated plasma membrane vesicles from

MDR cells (Cornwell et al., 1986A,B). Since vinblastine is known to bind to P-glycoprotein (Safa et al., 1986) this suggests that verapamil can also bind to the P-glycoprotein. Cornwell et al. (1987) have also shown that verapamil is capable of competing with a variety of cytotoxic drugs of the MDR family for the binding sites on P-glycoprotein. Furthermore recent studies have shown that the phosphorylation state of the P-glycoprotein may modulate its function. Studies using an MDR human leukaemia cell line indicate that agents which are capable of restoring drug sensitivity, such as verapamil, cause a significant increase in phosphorylation of the P-glycoprotein (Hamada et al., 1987).

Alternative mechanisms of action for verapamil have been proposed. For example, it might exert its effect by altering membrane trafficking (Beck, 1987). Indeed recently Sehested et al. (1987B) have shown that the ability of verapamil to restore chemosensitivity relates to disruption of vesicular traffic. Warr et al. (1986) suggested that collateral sensitivity of MDR cells to modulators such as verapamil might relate to a general membrane perturbing effect. In this regard it is worth noting that relatively high molar concentrations of "modifier" are required for an effect. These relatively high concentrations are also relevant to the application of agents identified as resistance modifiers in the laboratory, to the clinical setting. In particular, a major obstacle in the clinic is that of achieving plasma levels in patients which are similar to those known to be most active in the laboratory (See Chapter 7).

Other Calcium Antagonists

Other calcium antagonists have also been shown to be active as resistance modifiers in the laboratory. Tsuruo and co-workers have demonstrated the circumvention of resistance to both vincristine and doxorubicin in vitro and also in vivo using a variety of calcium antagonists including nifedipine and diltiazem (Tsuruo et al., 1983A,B,C,D). More recently bepridil has been shown to enhance doxorubicin cytotoxicity in both anthracycline-resistant and -sensitive cell lines. Moreover this effect was seen in vitro at levels that may readily be achieved in the clinic (Schuurhuis et al., 1987).

Recently an analogue of tiapamil, N-(3,4-dimethoxyphenethyl)-N-methyl-2-(2-naphthyl-m-dithane-2-propylamine) (DMDP), was shown to have a greater resistance modifying effect than verapamil in a doxorubicin resistant P388 leukaemia cell line (Radel et al., 1988). It exerted its modulating effect at half the concentration of verapamil. An important aspect of this work was the demonstration that a 24 hour exposure to DMDP with a short exposure to doxorubicin resulted in the same cytotoxicity as a 24 hour exposure to both agents. Moreover the former regime resulted in prolonged intracellular retention of doxorubicin.

Quinidine

Quinidine has been shown to be active as a resistance modifying agent in laboratory tumour models (Tsuruo et al., 1984). It appears to be capable of reversing MDR and altering intracellular accumulation of vincristine and doxorubicin in both P388 leukaemia cell lines and in the human myelogenous leukaemia cell line, K562. However, the effect of quinidine in vitro in the above models is considerably less than that observed with calcium channel blocking drugs. Quinidine (like other antidysrhythmics) may produce its resistance modifying effect by perturbing the organisation of membrane lipids and so non-specifically disturb various membrane-bound enzymes which are believed to be influenced by the physical state of surrounding lipids (Surewicz & Jozwiak, 1983). Quinidine may also affect sub-cellular calcium distribution and thus decrease the free intra-cellular calcium level (Harrow & Dhalla, 1976). Quinidine is of particular interest as a resistance modifying agent because it is active in vitro at concentrations that are achievable in the clinic (Tsuruo et al., 1984).

Calmodulin Inhibitors

Calmodulin antagonists such as chlorpromazine and trifluoperazine have been shown to potentiate the cytotoxicity of vincristine and doxorubicin in vitro (Tsuruo et al., 1983D). This effect appears to relate to increased accumulation of cytotoxic drug

in resistant cells following incubation with calmodulin inhibitor (Tsuruo et al., 1982). More recently Akiyama et al. (1986) demonstrated circumvention of multiple drug resistance in human cancer cells using a range of calmodulin inhibitors. Working with a human KB carcinoma cell line made resistant to colchicine, and exhibiting a typical MDR pattern of cross-resistance, they showed that thioridazine had the greatest sensitizing effect although trifluoperazine and chlorpromazine also had some sensitising effect. However, the calmodulin inhibitor, N-(6-aminohexyl)-5-chloro-1-naphthalene sulphonamide did not reverse MDR. Thus the effect seen with thioridazine, trifluoperazine and chlorpromazine appeared not to relate specifically to calmodulin inhibition. Once again the enhanced drug efflux seen in the MDR cells was inhibited by thioridazine. It has been speculated that calmodulin antagonists (as indeed verapamil) may produce their effects by acting on lysosomal function. Both verapamil (Akiyama et al., 1984, 1985B) and thioridazine (Kuratomi et al., 1986) have been shown to affect lysosomal function and delay the degradation of ligands such as low density lipoproteins and epidermal growth factor in lysosomes.

Cyclosporins

Slater and colleagues (1986A,B) have shown that the immunosuppressive drug cyclosporin A acts as a modifier of resistance to daunorubicin and vincristine in Ehrlich ascites carcinoma in vivo and in human acute lymphatic leukaemia in vitro. Recent studies of cyclosporin A in a highly resistant small cell lung

cancer cell line confirmed that this agent could considerably reduce the degree of resistance to doxorubicin and vincristine (Twentyman et al., 1987). A close correlation was noted between the immunosuppressive properties of natural cyclosporin analogues and their ability to modify drug resistance. Subsequently, Twentyman (1988) showed that some non-immunosuppressive synthetic cyclosporin analogues are also highly effective as modifiers of resistance to doxorubicin and vincristine in the MDR small cell lung cancer cell line, H69LX4. Cyclosporins themselves are generally growth inhibitory (Saydjari et al., 1986). Nonetheless these synthetic non-immunosuppressive cyclosporin analogues do present an exciting area of research as they appear to retain their resistance modifying activity at lower concentrations than cyclosporin A. This is an important observation because the plasma levels of cyclosporin A achievable in the clinic (1-2 ug/ml) (Kahan et al., 1983) are considerably lower than the levels required to produce significant modulation in vitro (5 ug/ml).

Amiodarone

A French group have published a report indicating that in a rat colon cancer model, amiodarone is more efficient than verapamil in enhancing the cytotoxic effect of 4⁺-deoxydoxorubicin (Chauffert et al., 1987). They also demonstrated that a 1 hour pre-treatment with amiodarone produced an increased resistance modifying effect, whereas verapamil pre-treatment did not. Levels of amiodarone achievable in patients (up to 7 uM after intravenous

dosing and up to 4 μM after oral dosing) are comparable to levels found to be maximally active in the laboratory (Chauffert et al., 1987; Mostow et al., 1984).

Thus it may be seen that there is great continuing interest in evaluating agents in the laboratory which may be suitable as resistance modifiers in the clinic.

Aims of the Study

The vast bulk of laboratory work which has investigated possible mechanisms of resistance and has evaluated the role of resistance modifying drugs has used in vitro derived resistant cell lines as models for drug resistance. However, it is difficult to be certain what relevance these laboratory derived models have to drug resistance in the clinical setting.

Therefore, the main aim of this thesis has been to establish cell lines from small cell lung cancer biopsies (primary and metastatic) taken from both untreated patients and from patients who had relapsed following chemotherapy. It was thereby hoped to obtain a group of cell lines with a range of drug sensitivities to use as the basis for studies of drug resistance in this tumour type. Since doxorubicin is one of the most active agents in the chemotherapy of small cell lung cancer, the chemosensitivity of the newly established SCLC cell lines to this cytotoxic drug was

measured in the first instance. In addition the effects of verapamil on the chemosensitivity of the cell lines to doxorubicin was assessed. Verapamil was selected as the resistance modifier as it is the agent which has been most widely investigated as a modulator, and already is used extensively in the clinic in patients with hypertension, ischaemic heart disease and cardiac dysrhythmias.

The three principle aims of this work can be summarised as follows:

- (1) To establish a number of SCLC cell lines from biopsies of patients before treatment and at time of relapse following chemotherapy.
- (2) To determine the sensitivity of the cell lines to doxorubicin and to investigate the activity of verapamil as a resistance modifier in these cell lines.
- (3) To evaluate the potential role of verapamil as a resistance modifier in the clinic in a large-scale randomised clinical study.

Layout of the Thesis

Each Chapter includes a self-contained Introduction and therefore details of the rationale behind the various stages of the work will not be discussed here.

Chapter 2 describes the establishment of 9 SCLC cell lines. Their detailed characterisation is presented in Chapter 3. The cell lines were established from biopsies of both untreated and treated, relapse patients. Of particular interest are a pair of cell lines established from a single patient, one pre-treatment and one post-chemotherapy from relapse tumour.

The growth characteristics of these cell-lines presented major problems in the study of growth and drug sensitivity. Since the cell lines all grow as non-adherent floating aggregates and have similar morphological characteristics to man-made tumour cell spheroids, the analytical methods used to study spheroid growth were applied to the SCLC cell lines. These studies are described in Chapter 4, which discusses the problems encountered with this approach.

Chapter 5 describes the adaptation of a tetrazolium dye-based chemosensitivity assay that can be used for both adherent and non-adherent cell lines. This assay proved suitable for use with the SCLC cell lines and Chapter 6 details the growth rates and chemosensitivities to doxorubicin of the cell lines. In addition the effects of verapamil on the chemosensitivities of the cell lines to doxorubicin is described in Chapter 6. Chapter 7 describes a large-scale multi-centre randomised trial of verapamil in addition

to chemotherapy in patients with SCLC.

The final conclusions of this work are presented in Chapter 8.

CHAPTER 2

ESTABLISHMENT OF SMALL CELL LUNG CANCER

CELL LINES IN VITRO

INTRODUCTION

Cells growing continuously in tissue culture (cell lines) have been widely used as laboratory models in an increasing range of scientific specialities. A number of workers have established SCLC cell lines (Carney et al., 1985A; Duchesne et al., 1987; Gazdar et al., 1980,1985; Pettengill et al., 1980). A major aim of this project was to establish SCLC cell lines in vitro, from both the primary tumour site and metastatic deposits, from patients before treatment and also from patients who had relapsed following chemotherapy. Hence it was hoped to establish cell lines with a range of chemosensitivities (Carney et al., 1983), in order to study the chemosensitivity patterns and effects of resistance modifiers in relatively sensitive and resistant SCLC cell lines in vitro.

A particular aim was to establish cell lines from the primary tumour (biopsied using fibre-optic bronchoscopy) or from a metastatic deposit before any chemotherapy was given. These patients could then be followed-up until they relapsed with recurrent tumour. Cell lines from such relapse tumour (either primary or metastatic) could then be established, and regarded as a model for drug resistance in

vitro where resistance had developed in the clinic.

Another aim of this project originally was to attempt to establish a number of clonal cell lines from a single tumour biopsy, immediately after explantation. This would provide information on the possible existence of sub-populations of tumour cells of varying chemosensitivity within a tumour.

Establishment of SCLC Cell Lines In Vitro

A large number of centres have already reported techniques for establishing SCLC cell lines in vitro. The first report came from Japan in the early 1970s (Oboshi et al., 1971). Towards the end of that decade and into the 1980s, there was a dramatic rise in the number of centres establishing SCLC cell lines. One of the most active in this regard has been John Minna's laboratory at the National Cancer Institute at Bethesda in the United States (Carney et al., 1985A; Gazdar et al., 1980, 1985). The major problems in establishing SCLC cell lines cited by this group and others (Baillie-Johnson et al., 1985; Bepler et al., 1987A,B,C; Duchesne et al., 1987; Pettengill et al., 1980) were the inadequacy of viable tumour material, infection, and difficulties associated with fibroblast overgrowth.

Three basic techniques have been used to try and overcome the problem of fibroblast overgrowth. They are the use of collagenase, the use of feeder-layers and the use of serum-free medium, with

appropriate supplements.

Collagenase is an enzyme that digests the collagen component of the extracellular matrix. In the presence of collagenase cells are unable to produce an extracellular matrix. Cells such as fibroblasts that are anchorage-dependent cannot proliferate in suspension. However many tumour cells exhibit anchorage-independent growth and are able to proliferate in the presence of collagenase. This approach has been used for small cell lung cancer biopsies (Pettengill et al., 1980) where agarose overlays were also used to prevent fibroblast attachment. Collagenase is also used for the enzymatic disaggregation of larger biopsies where physical disaggregation is inefficient. Recently Duchesne et al. (1987) described the use of enzymatic disaggregation in establishing small cell tumour biopsies in culture.

Feeder-layers have also been used to suppress fibroblast overgrowth. Feeder-layers may consist of STO mouse tumour cells exposed to Mitomycin-C, which acts as a cytostatic agent, arresting STO cell growth, but maintaining cellular metabolism. Ultimately the STO mouse tumour cells die, as the Mitomycin-C damages the cellular DNA. These Mitomycin-C treated STO mouse tumour cells recognise fibroblasts and this results in contact inhibition of fibroblast growth. However, epithelial human tumour cells do not exhibit contact inhibition and may continue to grow. Thus Mitomycin-C treated STO tumour cells used as a feeder-layer, suppress fibroblast overgrowth. In addition, because the drug-treated STO cells remain metabolically active, they may condition the medium in which the SCLC cells are

growing (see below). Various feeder-layer techniques have been used in the establishment of SCLC biopsies previously. Bergh et al. (1985) described the use of glial cells as feeder-layers and Gazdar et al. (1980) have co-cultured tumour cells with mouse fibroblasts. More recently the usefulness of feeder-layers has again been reported (Gazdar and Oie, 1986).

Serum-free medium has also been used to try and suppress fibroblast growth. Carney et al. (1981,1985A) described the use of serum-free medium in establishing SCLC cell lines in vitro. Such an approach proved to be particularly helpful in stemming fibroblast overgrowth at an early stage after explantation. However, it has been noted that for continued SCLC growth in vitro, serum supplementation is often required (Carney et al., 1981). The advantages and disadvantages of serum-containing and serum-free medium in establishing SCLC cell lines are reviewed later.

In an attempt to maximise the chance of successfully establishing cell lines, conditioned medium has also been used. Gazdar et al. (1980), described the use of conditioned medium in SCLC in vitro in some detail and showed that only 2 of 21 tumour specimens plated out in serum-supplemented medium yielded cell lines but 6 of 11 tumour specimens plated out in medium supplemented by conditioned medium grew. Subsequently Minna et al. (1982) reported that medium from all SCLC cell lines stimulated colony formation of other SCLC cell lines. More recently Carney et al. (1985A) described

the usefulness of conditioned medium in establishing SCLC cell lines.

These various techniques were investigated in an attempt to establish a panel of SCLC cell lines for studies of drug sensitivity and resistance modulation.

METHODS

Tumour Samples and Biopsy Techniques

Both primary and metastatic tumour samples and SCLC xenograft explants have been established in tissue culture.

Primary tumour was obtained via the fibre-optic bronchoscope. All bronchoscopies were performed by myself after a diagnosis of small cell lung cancer had already been established. After sedation with intravenous diazepam and topical anaesthesia with lignocaine (4%) the bronchoscope was passed via the nostril into the airways. As many biopsies as possible (up to a maximum of 10) were taken with alligator forceps, and transferred to culture medium (see below). The patients, all of whom had given informed consent, invariably tolerated this procedure well and there were no complications.

Biopsies from metastatic deposits were obtained with myself in attendance by conventional surgical techniques under strict aseptic conditions and with appropriate local/general (for cervical lymph nodes) anaesthesia. Pleural effusions were aspirated by myself according to conventional techniques, under local anaesthesia (lignocaine, 2%) and appropriate asepsis.

SCLC tumour xenografts established in athymic nude mice were removed surgically from an ether-anaesthetised animal. One part of the tumour was preserved in formal saline for subsequent histological examination to confirm that the tumour was SCLC. Another part was

transferred to appropriate culture medium and processed as described below.

All solid tumour tissue samples were transferred to 20 mls culture medium (Roswell Park Memorial Institute [RPMI] 1640 with 10% v/v foetal bovine serum) with antibiotics (penicillin [250 units/ml], streptomycin [250 ug/ml], gentamycin [50 ug/ml]), and an antifungal agent (amphotericin [2.5 ug/ml]), in a sterile universal container. Biopsies were then immediately transported to the laboratory and processed. Pleural fluid was collected in large volume (500 mls) sterile plastic containers containing preservative-free heparin as an anti-coagulant (final heparin concentration 0.2 mg%) and transported immediately to the laboratory.

Media

Roswell Park Memorial Institute (RPMI) 1640 Medium (Gibco, Paisley, U.K.) was the culture medium generally used. A monolayer SCLC cell line, LS112ST, was established and this was maintained in Waymouth's medium (Gibco, Paisley, U.K.). Culture medium was supplemented with glutamine (2mM), and foetal bovine serum (2.5% - 10% v/v) and antibiotics (penicillin 250 units/ml, streptomycin 250 ug/ml, gentamycin 50 ug/ml), and an antifungal agent (amphotericin 2.5 ug/ml). If fibroblast growth became excessive then medium containing reduced serum concentrations was used. This comprised RPMI 1640 as described above supplemented with 2.5% v/v foetal

bovine serum, with SIT additive (Sigma Co., Poole, Dorset) containing final concentrations of selenium (5 ng/ml), insulin (5 ug/ml), and transferrin (5 ug/ml) (RPMI 2.5% SIT).

Disaggregation

Samples were freed of fat and fibrous tissue and the tumour tissue was carefully dissected in a Petri dish using crossed scalpel blades to obtain fragments of 1 mm x 1 mm. The smaller bronchial biopsy specimens required less physical disaggregation than metastatic skin and lymph node deposits or tumour xenografts. The disaggregation was performed in a sterile laminar flow hood.

Pleural effusions were examined and if macroscopically bloody or if many red cells were seen on microscopy, the fluid was centrifuged (200 g for 5 minutes) through an equal volume of Ficoll-Paque (Pharmacia Ltd., Milton Keynes, U.K.). The red cell pellet was discarded and the cells at the interface resuspended in 4 volumes of phosphate buffered saline (PBS) and centrifuged (200g, 5 minutes). The resulting cellular pellet was resuspended in a small volume (5mls) of culture medium as described below.

The disaggregated tumour material was resuspended in 5 mls of RPMI medium containing 10% v/v foetal bovine serum (FBS) with antibiotics (as above) and glutamine (2mM) [RPMI 10%] and equilibrated with 2% CO₂ in air and incubated at 37°C in a humidified atmosphere. Generally at least two separate 25cm² flasks (Nunc);

Gibco, Paisley, U.K.) with tumour material at relatively higher and lower cell densities were established. Cells were examined 2-3 times per week and the medium changed 1-2 times per week.

Collagenase

Some specimens were treated with collagenase. When used, samples were dissected into small fragments and the resulting tissue divided into two parts. One part was put into a 25 cm² flask (Nunc; Gibco, Paisley, U.K.) in 5 mls of RPMI 10%, as described above. The other part was suspended in collagenase (Boehringer Mannheim, Lewes, U.K.) 200 units/ml in RPMI 10% (5 mls) and incubated for 24 hours. After 24 hours the sample was centrifuged (200 g for 5 minutes), the medium containing collagenase removed, the sample was washed with sterile PBS and resuspended in 5 mls of RPMI 10%.

STO Feeder Layers

For preparation of the feeder-layers exponentially growing STO fibroblasts were treated with Mitomycin-C. When used, STO fibroblast cells growing in 25 cm² flasks, almost at confluence, were exposed to Mitomycin-C, at a concentration of 2 ug/10⁶ cells (i.e. 20 ug/25 cm² flask), for 24 hours. The monolayer was then washed twice with PBS, and could then be used as a feeder layer.

Conditioned Medium

Conditioned medium was obtained from either more active flasks of the same cell line, from other actively growing SCLC cell lines or from the longer established SCLC cell line, NCI-H187. This was a gift from Dr D. Carney (NCI, Bethesda). In all cases such conditioned medium was filter-sterilised using a 0.22 micron pore filter (Millipore U.K. Ltd., London, England) to avoid cellular cross-contamination.

Maintaining Cells in Culture

In those cases where there was evidence of cellular proliferation, the medium required to be changed up to 3 times weekly. In the other cases half of the medium was replaced weekly. After 1-2 months in culture, the developing cell lines were transferred to medium without antibiotics.

Specific Experiments

LS111 "Cloning"

Actively growing LS111 aggregates were disrupted mechanically and serially diluted (from 1:20 to $1:2 \times 10^5$) and plated out across a 96-well plate (Linbro; Flow Laboratories, Rickmansworth, U.K.).

The plate was examined microscopically. The two rows (16 wells) in which aggregates at a dilution of $1:2 \times 10^{-4}$ were plated had 3 individual wells with a single small aggregate in each. These were monitored carefully thrice weekly and fed weekly over the next 2 months. Only one aggregate ultimately started to grow and expand. The aggregates from this well were transferred to a single well of a 24 well plate (Linbro; Flow Laboratories) and after a further few months they were transferred to a 25 cm² flask in 5 mls of RPMI 10% and grown up. This process yielded the sub-line, LS111 "Clone".

LS129

In order to attempt to establish a number of cell lines from a single tumour biopsy a 24 well plate (Linbro; Flow Laboratories) was pre-prepared with Mitomycin-C-treated STO fibroblasts (see above). The biopsy material was then processed as usual but instead of being placed in 25 cm² flasks, was plated out in small (1/24) portions onto the feeder layer. The wells were examined thrice weekly and fed once weekly for three months.

RESULTS

A total of 21 SCLC biopsies were processed during the period January 1986 to May 1988. The details of these biopsies and processing are shown in Table 2.1. These include 12 metastatic deposits, 7 primary bronchial biopsies and 2 xenograft SCLC tumours which gave rise to 6, 1 and 2 cell lines respectively.

It may be seen from Table 2.1 that 9 cell lines have been successfully established from both primary and metastatic tumour, from both untreated and previously treated patients and from patients with relapse disease. There appeared to be no relationship between source or history of biopsy and the ability to establish a cell line in vitro. Similarly there was no clear benefit from the use of collagenase, STO feeder-layers or conditioned medium.

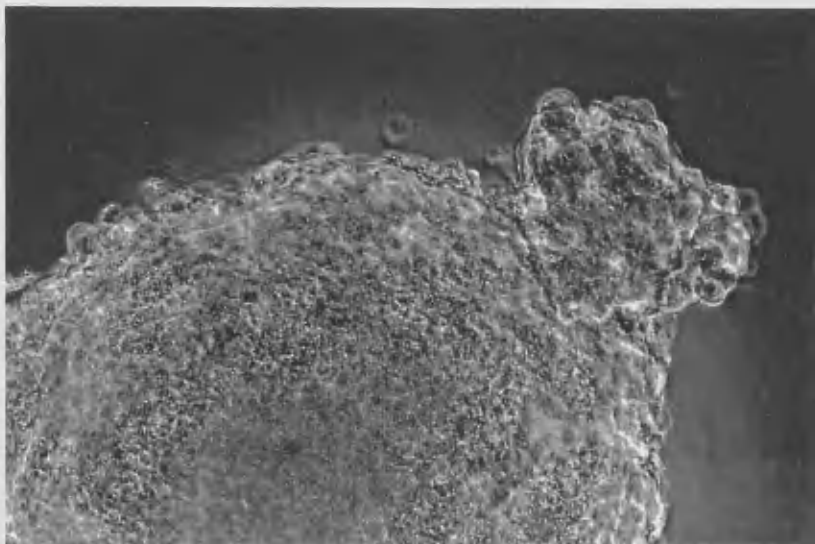
It should be noted that in most cases it took many months for the explanted SCLC material to proliferate into a cell line. Often the tumour aggregates would initially simply adhere to the base of the flask. Over a period of weeks fibroblasts would proliferate as outgrowths along the base of the flasks and after many weeks small clumps of tumour cells would grow out from the nests of biopsy material enmeshed in the fibroblast monolayer. Ultimately, these tumour cells would break off into the medium as free floating tumour clumps (Plate 2.1). These could be harvested and transferred to other flasks and ultimately yielded continuously growing SCLC cell lines in 9 cases. Although morphology varied between cell lines, all

Biopsy	Source	History	Collagenase	STO Feeder Layer	Conditioned Medium	Summary
LS106	Bronchial Biopsy	Pre-therapy, subsequent PR	No	No	Yes	Cell line
LS107	Skin Metastasis	Pre-therapy	Yes	No	Yes	Failed
LS108	Pleural Effusion	Pre-therapy	No	Yes	Yes	Failed
LS109	Bronchial Biopsy	Pre-therapy	Yes	No	Yes	Failed
LS110	Bronchial Biopsy	Pre-therapy (same patient as LS106)	Yes	No	Yes	Failed
LS111	Neck Node	Pre-therapy, subsequent PR/CR	Yes	No	Yes	Cell line
LS112	Skin Metastasis	Pre-therapy, no treatment	Yes	No	Yes	Cell line
LS124	Bronchial biopsy	Pre-therapy	Yes	Yes	No	Failed
LS126	Lymph Node	Pre-therapy	Yes	Yes	No	Failed
LS127	Bronchial Biopsy	Pre-therapy (same patient as LS126)	Yes	No	No	Failed
LS128	Bronchial Biopsy	Pre-therapy	Yes	Yes	No	Failed
LS129	Bronchial Biopsy	Pre-therapy	Yes	Yes	No	Failed
LS131	Skin Metastasis	Post-therapy, relapse after PR (same patient as LS106)	Yes	No	No	Failed
LS132	Neck Node	Post-therapy, relapse after PR	Yes	No	No	Failed
LS133	Pleural Effusion	Post-therapy, unresponsive	No	No	No	Failed
LCPH2	Mouse xenograft	Post-therapy, relapse	No	No	No	Cell line
LCPH3	Mouse xenograft	Post-therapy, relapse (same patient as LCPH2)	No	No	No	Cell line
LS263	Neck Node	Post-therapy, unresponsive	No	No	No	Cell line
LS274	Skin Nodule	Pre-therapy, subsequent PR	No	No	No	Cell line
LS277	Skin Nodule	Pre-therapy, subsequent CR	No	No	No	Cell line
LS310	Axillary Node	Post-therapy, relapse after PR (same patient as LS274)	No	No	No	Cell line

Table 2.1

Sources of biopsies and methodology for establishing SCLC cell lines.

A



B

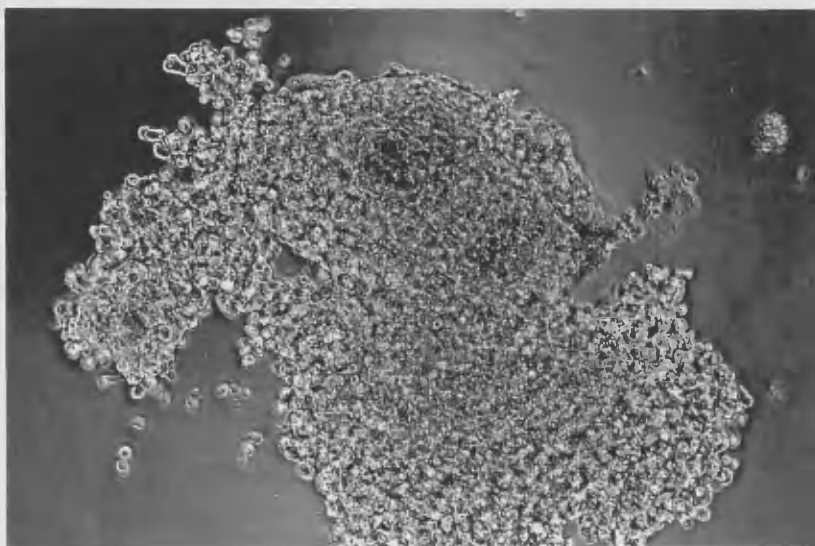


Plate 2.1

Viablc clumps of tumour cells at an early stage (weeks after explantation) in tissue culture. (A, LS106; B, LS112).

grew as floating aggregates in vitro (Plate 2.2).

In the case of cell line LS112, two distinct sub-populations of tumour cells developed. One comprised the typical SCLC floating aggregates, LS112 FL (Plate 2.3A). These, however, tended to adhere to the base of the flask and these adherent tumour aggregates then produced a distinct monolayer SCLC sub-line, LS112 ST (Plate 2.3B). The propensity to form an SCLC monolayer by LS112 could be enhanced by culture in Waymouth's medium with 10% v/v FBS.

Clinical History of Cell-Lines

Cell Line LS106

56 year old male presented with haemoptysis. Chest x-ray showed collapse of the left lung and bronchoscopy showed proximal tumour in the left main stem bronchus. Histological examination demonstrated small cell carcinoma of intermediate cell type. Bronchial biopsies yielded cell line LS106. Subsequent staging with whole body Gallium scan and ultrasound examination indicated that disease was confined to the thorax. He received 4 cycles of induction chemotherapy with cyclophosphamide, doxorubicin, vincristine and etoposide with verapamil and restaging indicated a partial response. He therefore received a course of consolidative mediastinal irradiation (3,000 cGy. given in 10 fractions over 2 weeks). He remained stable for a few months but then complained of malaise, weight loss and an abdominal wall swelling. Investigation confirmed both local (thoracic)

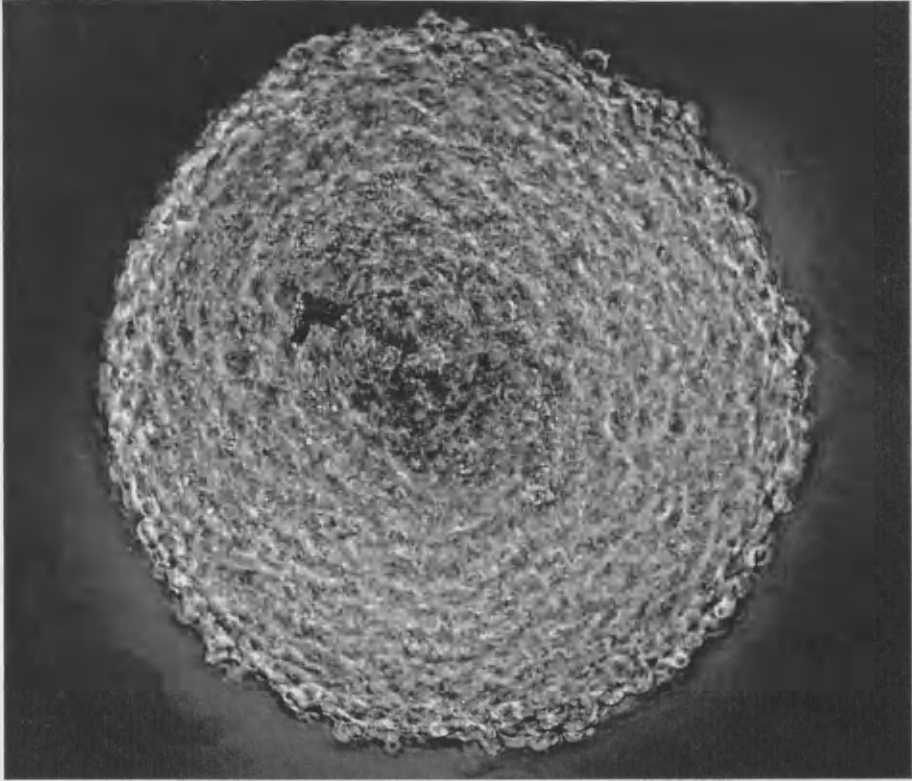
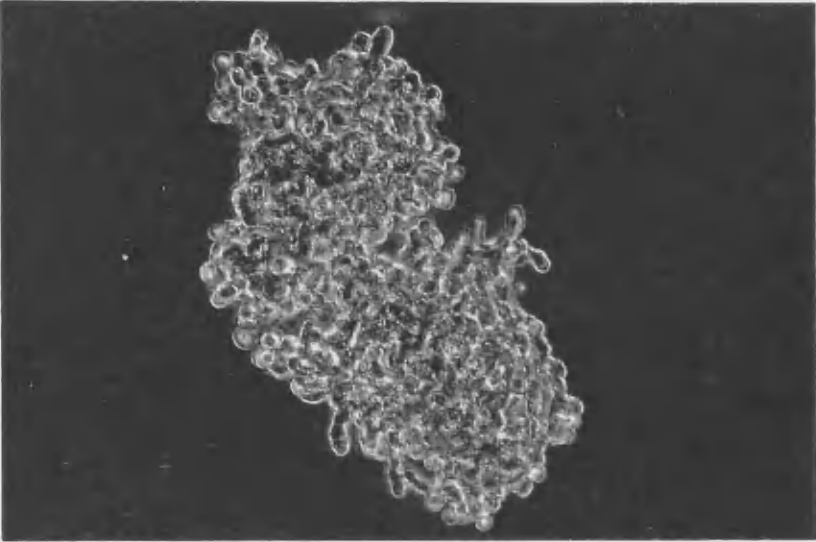


Plate 2.2

Cell line LS111 grows as tight, non-adherent aggregates of tumour cells.

A



B

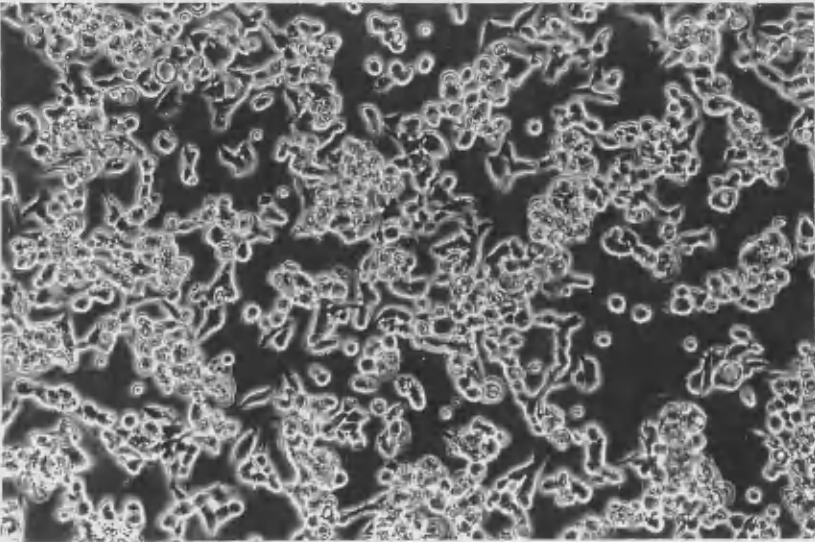


Plate 2.3

Two cell populations derived from biopsy LS112.

LS112FL grows as loose non-adherent aggregates of cells (A) and LS112ST, a monolayer sub-line established in selective culture medium (B).

relapse and abdominal wall metastases. This was biopsied and established in tissue culture (LS131) but unfortunately did not yield a continuous cell line. His condition deteriorated and he died with extensive metastatic disease 8 months after presentation. Cell line LS106 is therefore representative of a partially chemosensitive tumour in the clinic.

LS111

59 year old female smoker presented with a right sided supra-clavicular swelling. Chest x-ray showed a right hilar mass. Bronchoscopy revealed right sided tumour, histological examination of which revealed the presence of small cell anaplastic carcinoma. Lymph node biopsy confirmed the presence of SCLC, and this yielded cell line LS111. Further investigation, including liver ultrasound indicated that she had limited disease. There was a past history of ischaemic heart disease. She showed an excellent response to her first cycle of induction chemotherapy with cyclophosphamide, doxorubicin, vincristine and etoposide with dramatic regression of the lymphadenopathy and a marked reduction in the right hilar mass on the chest x-ray. She developed neutropenia from which she made a full recovery and was re-admitted for her second cycle of chemotherapy. She died suddenly of a presumed dysrhythmia thought to be secondary to a recent myocardial infarction demonstrated at post-mortem, 5 weeks after diagnosis. Cell line LS111 is an example of a chemosensitive small cell lung tumour in the clinic.

LS112

65 year old male smoker who presented with headaches and skin nodules. Biopsy of the latter demonstrated evidence of small cell anaplastic carcinoma. Isotope brain scan suggested cerebral metastases, and chest x-ray demonstrated a central mass lesion. His condition was considered too frail and his disease too extensive for active treatment and he died comfortably a few weeks after presentation. Skin biopsy yielded cell line LS112, which represents a very advanced small cell lung tumour in the clinic.

LCPH2 and LCPH3

33 year old male smoker who presented with a 5 month history of general malaise and weight loss. He had extensive skin nodules at presentation, with axillary lymphadenopathy and a large right hilar mass on chest x-ray. Biopsy indicated extensive SCLC. He responded extremely well to initial chemotherapy with cyclophosphamide, doxorubicin, etoposide, vincristine and methotrexate. There was marked resolution of his skin and nodal metastases. His chest x-ray returned to normal and re-staging bronchoscopy showed normal endobronchial appearances. Two months after restaging he developed extensive lymphadenopathy which was shown to be due to recurrent small cell tumour. LCPH2 was established from a mouse xenograft of tumour from the supraclavicular gland mass at time of initial relapse. His relapse was treated with a phase I drug (CB3717). However, he then developed SVC obstruction

and was treated with mediastinal irradiation. Although the SVC obstruction responded to radiotherapy, his peripheral disease continued to progress and he was treated with a single course of the alkylating agent, TGU. LCPH3 was established from a mouse xenograft tumour from a chest wall metastasis biopsied at a time when his disease was progressing relentlessly despite additional chemotherapy with TGU. His disease continued to advance with the development of liver metastases, and he was therefore treated with a course of Cholorambucil, Etoposide and Prednisolone. There was no response to this therapy and he died 7 months after his initial response to chemotherapy. Cell lines LCPH2 and LCPH3 are therefore representative of small cell lung cancer progressing in the clinic.

LS263

53 year old male smoker presented with cough and dyspnoea. Chest x-ray showed a right upper lobe mass with mediastinal lymphadenopathy. Bronchoscopy showed tumour occluding the right main stem bronchus. Histological examination demonstrated small cell anaplastic carcinoma. He was treated with 3 cycles of cyclophosphamide, doxorubicin, vincristine and etoposide with verapamil. Despite this he developed progressive thoracic disease with total collapse of the right lung and he developed right-sided supraclavicular glands. This gland mass was biopsied and yielded cell line LS263. Despite treatment with ifosphamide and carboplatin and local radiotherapy his disease progressed and he died 4 months after

diagnosis. His tumour therefore appeared intrinsically resistant in the clinic.

LS274/LS310

61 year old male smoker presented with right chest wall pain, increased dyspnoea, anorexia, weight loss and malaise. He was found to have extensive skin nodules and neck and groin lymphadenopathy. Biopsy of a skin nodule demonstrated metastatic small cell lung cancer and yielded cell line LS274. Chest x-ray showed a bulky right hilum and right paratracheal lymphadenopathy. Bronchoscopy demonstrated tumour in the right upper lobe, biopsy of which confirmed primary SCLC.

He was treated with 4 cycles of cyclophosphamide, doxorubicin, vincristine and etoposide, without verapamil. He showed a partial response to chemotherapy. The right hilar bulkiness on chest x-ray resolved but the paratracheal shadowing persisted. There was complete resolution of the neck and groin lymphadenopathy and partial resolution of the skin nodules. A residual nodule, although shrunk from 4 cm x 3 cm to 0.5 cm x 0.5 cm, was biopsied after completion of chemotherapy. Histology demonstrated persisting viable small cell tumour cells. This biopsy yielded cell line LS310. He was reluctant to consider further active treatment and a few months later developed cerebral metastases which progressed rapidly despite palliative brain irradiation. He died 6 months after diagnosis. It may be seen that cell line LS274 represents a partially sensitive tumour in the clinic.

It is possible that cell

71

Λ line LS310 may be regarded as being representative of resistance that had developed in the clinic.

LS277

62 year old smoker with past history of Crohn's disease. Presented with left anterior chest pain and increased cough. There was clinical and radiological evidence of left lower lobe collapse. Bronchoscopy showed tumour in the left lower lobe, biopsy of which revealed SCLC. An abdominal wall nodule was noted, and biopsy of this demonstrated metastatic SCLC. This material yielded cell line LS277. There was no other evidence of metastases and she was treated with 4 cycles of cyclophosphamide, doxorubicin, vincristine and etoposide with verapamil. She showed a complete response to treatment radiologically and the sub-cutaneous nodule regressed almost completely. Restaging bronchoscopy showed no evidence of residual tumour. She was treated with mediastinal irradiation. She developed cerebral metastases and died 6 months after diagnosis. Cell line LS277 therefore represents a pre-treatment biopsy from a patient who had a chemosensitive tumour.

Results of Specific Experiments

LS111 "Cloning"

After two months one individual aggregate had divided sufficiently to allow it to be transferred to be grown on. Unfortunately only a single "clone" was established from LS111 and thus comparisons between different "clones" has not been possible.

LS129

The attempt to establish individual lines from an early explant has been described in "Methods" above. Over the next few months any viable tumour material that was present died. This primarily related to paucity of tumour material at outset.

DISCUSSION

Nine new small cell lung cancer cell lines have been successfully established in tissue culture. Their detailed characterisation is described in the next chapter. Overall this represents a success rate of 43%. Although this is not as high as has been previously reported (Carney et al., 1985A) it should be noted that the first 7 biopsies were processed in the first 3 weeks of the study. Indeed there was a learning curve evident in the processing of samples (success rate of 21% for the first 14 samples processed in 1986, rising to 100% in the more recently handled samples; LCPH2 and LCPH3 (1987) and LS263, LS274, LS277 and LS310 in 1988).

Adequacy of tumour material remains the most crucial factor in establishing SCLC cell lines. This is particularly the case in biopsies of primary tumour obtained via the fibre-optic bronchoscope. One continuous cell line has been successfully established in this way. Although a number of other workers have reported the establishment of SCLC cell lines from primary lung tumour this has either been from resected lung (ante- or immediately post-mortem) (Carney et al., 1985A) or from specimens taken via the rigid bronchoscope (Postmus et al., 1988) which allows larger biopsies to be taken but has the disadvantage of requiring general anaesthesia. The difficulty of establishing SCLC cell lines from primary tumour is confirmed by the large number of authors who have been unable to establish SCLC cell lines from primary tumour (Baillie-Johnson et al., 1985; Duchesne et

al., 1987; Pettengill et al., 1980). In a series of 50 cell lines Carney et al. (1985A) described 6 established from primary lung tissue, but these were all derived from material from a single tumour biopsy.

The difficulties of establishing primary SCLC tumours in culture underline the importance of obtaining adequate tumour material at outset. In retrospect therefore the division of biopsy material to treatment with or without collagenase and to co-culture with feeder-layer or not may have been erroneous as this will have reduced the tumour density in any given flask. This impression is corroborated by the higher success rate of establishing cell lines from more recent biopsies, which were not sub-divided at time of explantation.

The decision to plate initial biopsy material in culture medium containing 10% v/v foetal bovine serum was based on a number of premises. Most early researchers and a number of recent workers involved in establishing SCLC cell lines used medium containing serum (Bergh et al., 1985; De Leij et al., 1985; Gazdar et al., 1980; Pettengill et al., 1980). Moreover, although Carney et al. (1981, 1985A) have described the use of serum-free medium in successfully establishing SCLC cell lines, they did indicate that serum supplementation was often required for continued SCLC growth in vitro especially after the first few months (Carney et al., 1981). Indeed, a recent study comparing serum-free and serum-supplemented medium by Carney et al. (1985A) indicated that although 22 out of 31 SCLC biopsies could be established in serum-free medium, 19 out of 31 could be established in serum-containing medium. Thus

almost as many lines could be established in serum-containing medium as serum-free medium. Therefore, the use of serum-free medium based on earlier work by Hayashi and Sato (1976), and subsequently elaborated by Bottenstein et al.(1979) which described the addition of hydrocortisone, insulin, transferrin, estradiol and selenium to serum-free culture medium, and which had proved particularly helpful in stemming fibroblast overgrowth at an early stage after explantation was not routinely employed in this study. Similarly other trace substances previously employed as possible additives (Minna et al., 1982; Simms et al., 1980) were not routinely used in this study. Although the possibility that serum contains inhibitory factors to SCLC cells has been raised (Gazdar and Oie, 1986), this did not appear to be the case in this study.

The major disadvantage with plating the explant into serum-containing medium related to overgrowth of fibroblasts. Generally fibroblasts did develop and grow in the flasks containing explanted biopsy material. Initially often this proved helpful as the fibroblast monolayer acted as a bed for the tumour material to settle onto and form nests of tumour. However, in the case of the first biopsies handled, the fibroblasts often overgrew to the detriment of the tumour cell population. In an attempt to overcome this Mitomycin-C-treated STO mouse fibroblasts were used for two reasons - firstly to suppress the growth of fibroblasts from the biopsy itself and secondly in the hope that the feeder layer would condition the medium especially in the early stages after explantation (see also below).

However, the use of feeder-layers did not prove helpful in establishing new SCLC cell lines. This was partly because the mouse fibroblasts sometimes continued to grow and ultimately became detached from the base of the flask, and partly because the tumour material did not always settle onto the monolayer. After biopsy LS129 the use of feeder-layers was therefore discontinued. This resulted in greater rather than reduced success rates for establishing cell lines, probably because the biopsy material was not sub-divided between so many flasks at the time of explantation.

The other approach used to suppress fibroblast overgrowth was the use of collagenase. Generally the effectiveness of collagenase proved disappointing. Sometimes it appeared somewhat toxic to the tumour cells as shown by the healthier condition of the tumour cells in flasks which did not contain collagenase. Recently therefore (since biopsy LS133) the use of collagenase was discontinued. This has allowed the initial explanted material to be maintained at high cell density in just one or two 25 cm² flasks, and this approach appears to have been more fruitful.

In contrast to the disappointing results with the use of feeder-layers and with collagenase, the use of conditioned medium proved to be helpful, although not essential. Carefully filter-sterilised conditioned medium both from other longer established SCLC cell lines and from more active flasks of the same cell line was particularly useful in the early stages after explantation. At this stage biopsy material contains a relatively small number of metabolically active cells

and so the culture medium remains relatively alkaline, despite equilibration with 2% CO₂ in air. Conditioned medium can therefore be helpful in adjusting the pH of the culture medium (although this could also be achieved by the use of HEPES buffer), but more importantly may supply growth factors to stimulate tumour cell growth (Minna et al., 1982).

It is worth reiterating at this stage that SCLC biopsies can take many months to develop into cell lines. This slowness to generate tumour lines has been noted by many workers over the years (Carney et al., 1985A; Oboshi et al., 1971; Ohara & Okamoto, 1977).

Specific Experiments

An aspect of the initial project was to try and isolate separate cell lines from a single biopsy at an early stage, in order to determine whether a single SCLC biopsy could give rise to a range of phenotypically different cell lines. Although theoretically this concept appeared attractive, in realistic terms it was a mistaken approach. Indeed one biopsy (LS129) which was plated out at time of explantation into a 24-well plate which had been pre-prepared with feeder-layer failed to show any active growth. On the other hand once a biopsy sample had become established in tissue culture it was possible to isolate a separate cell line from a single floating aggregate, as was the case with LS111 and the sub-line LS111 "Clone". This of course is not a true clone, but given the problem of successfully

completely disaggregating individual SCLC aggregates it remains the model most like a clone realistically achievable. The similarities and differences between LS111 and its derivative LS111 "Clone" will be discussed in Chapter 3.

Summary

Nine new SCLC cell lines have been established from a total of 21 small cell lung cancer biopsies. Although conditioned medium was sometimes helpful in establishing SCLC cell lines, a large amount of tumour tissue in the initial biopsy was certainly helpful. The reduced tumour cell density which resulted as a consequence of sub-dividing biopsy material to treatment with and without collagenase and as a consequence of further sub-dividing material into flasks with and without feeder layers (and occasionally also into flasks containing different media) probably explains the relatively disappointing early results from this work. These new cell lines will form the basis for the laboratory studies of drug resistance in SCLC in the later part of this thesis. The characterisation of the 9 newly established SCLC cell lines is now described.

CHAPTER 3

CHARACTERISATION OF NEWLY ESTABLISHED SMALL CELL LUNG CANCER CELL LINES

INTRODUCTION

The original papers where detailed characterisation of newly established small cell lung cancer cell lines was undertaken came from the United States, from the National Cancer Institute, Bethesda (Gazdar et al., 1980) and Dartmouth Medical School (Pettengill et al., 1980). However since that time there has been an enormous increase of interest in the study of the biology of SCLC and a number of European Centres (Baillie-Johnson et al., 1985; Bergh et al., 1982, 1985; De Leij et al., 1985) have reported their experience establishing and characterising SCLC cell lines in vitro.

Classification of SCLC into Classic & Variant Sub-Groups

A range of characteristic features of small cell lung cancer have been identified. Recently more detailed studies of established cell lines has allowed the identification of two broad sub-types of human SCLC cell lines in vitro, based on morphological, biochemical and genetic characteristics (Carney et al., 1985A,B; Gazdar et al., 1985). The so-called "classic" cell lines grow as tightly packed floating

cellular aggregates, have long doubling times and low colony forming efficiencies and have a distinctive biochemical profile, with the presence of L-dopa-decarboxylase (DDC), bombesin-like immunoreactivity (BLI), neurone specific enolase (NSE), and high concentrations of the brain iso-enzyme of creatine kinase (CK-BB). On the other hand the so-called "variant" cell lines grow as rather loosely-packed aggregates, have more rapid doubling times and higher colony forming efficiency. They also have reduced or absent DDC and BLI and have lower concentrations of NSE. These variant cell lines demonstrate amplification of the C-myc oncogene and exhibit relative radio-resistance (Carney et al., 1983).

The Heterogeneity of Lung Cancer

Various features have been reported as characteristic for small cell lung cancer. However, with increasingly sophisticated methods being applied to the histological investigation of tumours, the fact that lung cancers are often made up of heterogeneous tumour cell populations (Dunnill & Gatter, 1986) is gradually gaining general acceptance.

Using electron microscopy Auerbach et al. (1982) found evidence of a second cell type in nine out of 35 cases of lung cancer. In examining 61 tumours that had been classified as adenocarcinoma Rainio et al. (1983) analysed mucus histochemistry and ultrastructure and found evidence of a second cell type in 18%. Saba et al. (1983)

using both electron microscopy and an immunoperoxidase method for detecting keratin noted both glandular and squamous differentiation in 17 out of 52 lung carcinomas. Furthermore, both McDowell & Trump (1981) and Horie & Ohta (1981) described carcinomas which on electron microscopy showed not only dense core granules but also foci of squamous differentiation together with areas of glandular differentiation associated with intra- and extra-cellular secretion of mucus. Even when using only light microscopy Hirsch et al. (1983) found 13% of 200 patients with lung cancer had features of more than one cell type in their tumour.

The advantage of immunocytochemistry in examining tumours is that a much larger sample is studied than with electron microscopy. Even so it is often very small when compared with the size of the whole tumour. Thus it might be expected that if tumours are examined by light and electron microscopy together with immunocytochemistry a higher percentage of mixed tumours would be found than if only one or two of these methods were used, and this has proved to be the case.

It is noteworthy that biochemical investigation of lung tumours may reveal ectopic hormone production by all classes of tumour. Merrill & Bondy (1982) described vasopressin in both small cell carcinoma and adenocarcinoma of lung and calcitonin in all varieties of lung cancer. Even more striking was the investigation by Berger et al. (1981) in which L-dopa-decarboxylase, histaminase, B-endorphin and calcitonin were measured in 50 lung cancers. They

found no single marker separated SCLC from the rest. Instead they discovered that there were quantitative differences between various histological types and concluded that the major forms of lung cancer "represented a continuum of differentiation with a common cell lineage".

Microscopic evidence of cytological heterogeneity is not found in all cases but this may well be due to the necessarily small sample examined in an individual tumour. Yet it seems probable that all lung cancers in fact contain elements of several cell types though in the majority one type dominates the histological picture. This has profound consequences when considering the histogenesis of lung cancer. Yesner & Carter (1982) in reviewing the pathology of lung tumours suggested that small cell carcinoma as well as squamous and adenocarcinomas were derived from primitive endoderm. They based this opinion on the observation that all varieties of lung cancer may occur within a single tumour and suggested that all lung cancers are part of a spectrum of differentiation.

In the past it has been suggested that small cell carcinomas were derived from Kultschitzky type cells in the bronchial mucosa which were considered to have a neuroectodermal origin. This view as to the origin of these cells is now somewhat discredited as the ultrastructural studies described above and others (McDowell & Trump, 1981; Raikhlin et al., 1979) have shown features characteristic of small cell carcinoma together with those of adenocarcinoma and squamous carcinoma in the same tumour.

Thus the evidence seems to point to all lung cancers being heterogeneous. In the case of SCLC this point has recently been highlighted by Engelholm et al. (1986). In an extensive study comparing the histological, electron microscopic and flow cytometric characteristics in a model system of metastatic SCLC (tumour biopsy material, cell lines and xenografts) it was found that although tumours in the various components of the model system preserved the characteristics of SCLC, there was evidence of inherent heterogeneity and genetic instability. Thus the main cell population in the patient tumour may not necessarily dominate in vitro or in nude mice. Similarly using flow cytometric DNA analysis it was found that the tumour cell sub-populations obtained by in vitro growth did not always reflect the composition of the original tumour. Indeed sub-populations, undetectable in the original tumour were found in a number of cell lines. Despite this heterogeneity, a number of features are said to be characteristic for SCLC and these are now reviewed.

Light Microscopy

The vast majority of small cell lung cancer cell lines grow as floating aggregates of cells in vitro (Carney et al., 1985A; Gazdar et al., 1980). It has been suggested that this phenomenon may relate to a lack of glycoprotein attachment factors (Baylin et al., 1982). The morphological characteristics have been used to sub-divide SCLC

cell lines into "classic" (i.e. tight clusters of cells) and "variant" (i.e. loose aggregates of cells) categories (Carney et al., 1985A).

The characteristic features of SCLC in tissue culture on light microscopy have been extensively described and are similar to those features seen in human small cell lung tumours (Matthews & Hirsch, 1981). In particular the cells are small with meagre cytoplasm. Nuclear chromatin may be seen distributed uniformly throughout the nuclei. Nucleoli are small and indistinct. Mitoses are numerous. Spindle and fusiform cells are also seen. These features should be present when newly established SCLC cell lines are examined by light microscopy.

Electron Microscopy

Small cell lung cancer specimens frequently exhibit characteristic features on electron microscopy. In particular the presence of membrane limited dense core granules (DCGs) has been widely reported in SCLC biopsies and cell lines. These are thought to represent a cellular neurosecretory function (Pettengill et al., 1980). More recently it has been reported that some variant SCLC cell lines do not have DCGs present (Carney et al., 1985A). Furthermore, De Leij et al. (1985) not only reported that DCGs were absent in 3 variant cell lines but commented on the fact that all showed features of epithelial differentiation, manifest by the presence of desmosomes and other less tight adhesion specialisations. Indeed

some cell lines were seen to have villous process formation, which in addition to apical tight junctions and secretory organelles are suggestive of adenodifferentiation. In this regard it is interesting to note that Mooi et al., (1986) were only able to demonstrate the presence of DCGs in 42 of 54 clinical SCLC cell specimens examined ultrastructurally. Another group has also reported features of epithelial differentiation on ultrastructural examination of clinical SCLC specimens (Iglehart et al., 1985).

Thus, a number of ultrastructural characteristics have been described in small cell lung cancer specimens and cell lines. Although the most characteristic feature is the presence of dense core granules, a number of other features may also be seen.

Immunocytochemistry

In 1981 a method for producing monoclonal antibodies with a specificity for human lung cancer was described (Cuttitta et al., 1981; Minna et al., 1981). In recent years, researchers have examined the significance of a wide range of antibodies in SCLC (De Leij et al., 1984; Postmus et al., 1988). Consequently there have been many reports of positive antibody reactions in small cell lung cancer. Many researchers have reported positive reactions with CAM 5.2 in SCLC. This is an antibody directed against low molecular weight cytokeratin and indicates an element of epithelial differentiation (Bernal et al., 1983; Makin et al., 1984; van Muijen et

al., 1984). The antibody directed against the human milk fat globulin antigen (HMFG2), expressed on epithelial cells (Burchell et al., 1983, 1984) and consequently also a marker for epithelial differentiation has also recently been reported to be positive in SCLC (Allan et al., 1987). On the other hand Bergh et al., (1984) did not find a positive reaction against cytokeratins in SCLC. To further complicate the issue some workers have demonstrated the presence of neurofilaments in SCLC (Bergh et al., 1984; Lehto et al., 1983). Recently Broers and associates have tried to resolve this issue by suggesting that classical SCLC cell lines have cytokeratins but no neurofilaments (Broers et al., 1985), whereas variant SCLC cell lines usually have neurofilaments and always contain vimentin intermediate filaments (Broers et al., 1986).

In terms of specific biochemical markers, antibodies directed against both bombesin (Cuttitta et al., 1985) and neurone specific enolase (NSE) (Sheppard et al., 1984) have given strong positive reactions, which are regarded as pointers to a diagnosis of SCLC.

More recently there has been increasing interest in the use of antibodies (both monoclonal and polyclonal) to try and detect other antigenic determinants in SCLC. A variety of antibodies have been identified and shown to give varying positive reactions to a range of antigenic determinants. These antibodies include the anti-SCLC antibody, 123C3, which probably identifies SCLC neurofilaments (Schol et al., 1988), an antibody directed against the C-myc oncogene product (Sikora et al., 1985), and EGFD10, an antibody thought to

react with the cytoplasmic portion of epidermal growth factor receptor (Gullick et al., 1986).

In summary a range of antibodies have been identified as useful in characterising SCLC. These include the epithelial markers CAM 5.2 and HMFG2 as well as the enzyme markers bombesin and NSE.

Cytogenetic Characteristics

Detailed cytogenetic analyses have been extensively undertaken, not only in fresh small cell lung tumour biopsy material but also in SCLC cell lines. The most widely reported specific chromosomal abnormality in SCLC cell lines and fresh tumour material is deletion of the short arm of chromosome 3, the so-called 3p deletion, first described by Whang-Peng and associates (1982) and subsequently reported by many other workers (Bepler et al., 1987B; De Leij et al., 1985; Falor et al., 1985; Postmus et al., 1988; Yunis, 1983).

In contrast Bergh et al. (1982) did not find this chromosomal abnormality in their cell lines. Similarly Wurster-Hill et al., (1984) reported that only 20% of SCLC cell lines demonstrated the 3p deletion. More recently deletion of a DNA sequence at the 3p 21 chromosome region has been identified using highly specific molecular biological techniques. This abnormality has now been noted in all major types of lung cancer (Kok et al., 1987). Thus it may be that by using more specific technology, the 3p deletion can be identified

in all SCLC specimens examined, as some are probably sub-microscopic. This loss of heterozygosity of 3p alleles has led to the suggestion that a recessive cancer gene, analagous to that proposed for the retinoblastoma locus on chromosome 13, may be located in this region. The loss of alleles in SCLC has also recently been demonstrated for loci on chromosome 13 (Harbour et al., 1988) and chromosome 11 (Shiraishi et al., 1987). Both are at or near sites of recessive cancer genes and their loss of heterozygosity has been observed in several types of tumour suggesting a role in tumour progression.

A host of additional chromosomal defects have been reported. Buys et al. (1983) described over-representation of chromosomes 13 and 20 and extra genetic material on 14p in 2 of 3 cell lines. One of their cell lines also had extra material at 12q similar to that previously reported by Bergh et al. (1982). Other abnormalities including deletion at 6q and an aberrant chromosome 12 have also been reported (Bepler et al., 1987B).

In summary, a variety of karyotypic abnormalities have been described in SCLC. The commonest, deletion of part of the short arm of chromosome 3, is probably present, at least sub-microscopically, in all cases.

Enzyme Activities

Since the first reports of hormone production by small cell lung cancer cell lines (Pettengill et al., 1977; Sorenson et al., 1981) a large number of substances have been reported to be secreted by SCLC in vitro, including calcitonin, ACTH, parathormone, glucagon, oestradiol, LH, somatostatin and neurotensin (Bepler et al., 1987B; Moody et al., 1985; Pettengill et al., 1985). Indeed Pettengill et al. (1985) not only demonstrated the presence of many hormones and enzymes in SCLC cell lines but also suggested that apart from differing concentrations between cell lines (e.g. ACTH levels in DMS53 and DMS79), concentrations might also vary in the same line over a period of time (e.g. calcitonin levels in cell lines DMS53 and DMS153). There has been particular interest in measurement of levels of the enzyme neurone specific enolase (NSE) in the laboratory (Bepler et al., 1987B; Marangos et al., 1982). Indeed NSE has been regarded as a marker enzyme for SCLC. However, until recently, those enzymes regarded as the most constant markers for SCLC were L-dopa-decarboxylase (L-DDC) (Baylin et al., 1980) and bombesin (Moody et al., 1981). Recent work has demonstrated the BB isoenzyme of creatine kinase (CK-BB) to be the most consistently present enzyme in SCLC cell lines and this should now be regarded as the definitive marker enzyme for SCLC (Gazdar et al., 1985). The MM isoenzyme of CK has also been identified in SCLC cell lines (De Leij et al., 1985; Postmus et al., 1988).

In summary, a host of substances produced by SCLC have been identified. The more widely used marker enzymes include NSE, bombesin, L-DDC and the BB isoenzyme of creatine kinase.

The newly established SCLC cell lines were therefore characterized by morphological, ultrastructural, immunocytochemical, cytogenetic and enzymatic analyses.

METHODS

Cell Lines

The cell lines examined are those whose establishment has been described in Chapter 2. NCI-H69 was used as a reference cell line for some of the studies. This cell line grows as floating aggregates in RPMI 1640 medium supplemented with 10% v/v foetal bovine serum and glutamine (2 mM). This cell line was kindly donated by Dr D. Carney, NCI, Bethesda, U.S.A.

Light Microscopy

Exponentially growing tumour cell aggregates, 24 hours after feeding with fresh medium, were washed once in phosphate buffered saline (PBS) and then fixed in formal saline (10% formaldehyde in PBS) for 24 hours. The fixed aggregates were embedded in paraffin wax, and four micron thick sections were cut, stained with haematoxylin and eosin and examined by light microscopy.

Electron Microscopy

Samples were taken from tissue culture and fixed in 4% glutaraldehyde buffered with Sorensen's phosphate buffer (pH7), post-fixed in osmium tetroxide in aqueous osmium, dehydrated with

absolute ethanol and embedded in Emix (Biorad Ltd., Watford). One micron sections from 3 to 4 blocks were stained with toluidine blue and examined by light microscopy (Philips EM 301G microscope). All grids were stained with uranyl acetate and lead citrate (Reynolds, 1963). All preparations were examined by transmission electron microscopy (EM) by at least two observers. Once a suitable area was identified on light microscopy 2 blocks were trimmed and ultra-thin sections were cut at a thickness of 50 nm and mounted on copper grids. Each observer looked for the presence or absence of membrane-limited dense core granules (neuro-secretory granules), tonofilament bundles, true desmosomes, and other less well developed adhesion specialisations, microvillous profiles, mucin granules and basal lamina formation. The abundance of specialised features was assessed and scored as one plus if they were unequivocally present but scanty in number, and as two plus, if their presence was prominent.

Immunocytochemistry

Cells were trypsinised if necessary (LS112ST) using 0.25% trypsin/EDTA solution, then pelleted and washed twice in phosphate buffered saline. The pellet was resuspended in human plasma and then thrombin added which caused a clot to develop. The pellet/clot was then fixed in buffered formal saline for 24 hours, before being processed to paraffin wax. Four to five micron sections were cut,

dewaxed in xylene and rehydrated. The sections were stained using a standard indirect immunoperoxidase method for CAM 5.2 (Becton Dickinson Ltd., Oxford, U.K.) and N.S.E., (Dako Ltd., High Wycombe, Bucks., U.K.) and using a standard peroxidase anti-peroxidase (PAP) method for the other antibodies [bombesin (NCI, Bethesda); HMFG2 (ICRF, London); EGFD10 (ICRF, London); C-myc (Ludwig Institute, Cambridge) and 123C3 (Sera Lab Ltd., Crawley Down, Sussex)]. In all cases endogenous peroxidase activity was blocked with freshly prepared 0.3% hydrogen peroxide in methanol, for 30 minutes. Slides were then washed in tris-buffered saline (pH 7.4). Antigen recognition was enhanced by trypsinisation (0.1% Sigma crude trypsin in 0.01% EDTA) prior to incubation with antibodies (except for NSE).

The epithelial marker HMFG2 was used as undiluted supernatant. The other epithelial marker, CAM 5.2 was used in a 1/10 dilution. The polyclonal antibody, neurone specific enolase was used in a dilution of 1/500. Bombesin and C-myc monoclonal antibodies were used at optimal dilutions (1/20 and 1/200 respectively). EGFD10 and the anti-SCLC antibody, 123C3 were both used in a 1 in 10 dilution. The antigenic determinants of the specific antibodies and their sources are detailed in Table 3.1.

Thereafter, for those sections stained using the PAP technique a standard 3-step peroxidase anti-peroxidase technique (Dako Ltd., High Wycombe, Bucks.) was applied. In such cases, background staining was reduced by the use of sheep serum (1:5 dilution). In all

ANTIBODY	SOURCE	ANTIGEN	REFERENCE
CAM 5.2	Becton Dickinson Ltd., Oxford, U.K.	Low molecular weight cytokeratin (epithelial cell marker)	Makin et al., 1984
NSE	Dako Ltd., High Wycombe, Bucks., U.K.	SCLC enzyme marker	Sheppard et al., 1984
HMFG2	Dr J Taylor-Papadimitriou Imperial Cancer Research Fund London	Human milk fat globulin antigen (expressed by epithelial cells)	Burchell et al., 1983, 1984
EGF (D10)	Dr W Gullick, Dr M Waterfield Imperial Cancer Research Fund London	Cytoplasmic portion of epidermal growth factor receptor	Gullick et al., 1986
Bombesin	Dr F Cuttitta and Dr D Carney National Cancer Institute Bethesda, U.S.A.	Neuropeptide	Cuttitta et al., 1985
123 C 3	Sera Lab Ltd., Crawley Down, Sussex, U.K.	SCLC neurofilament	Schol et al., 1988
C-myc	Dr E Evan Ludwig Institute for Cancer Research Cambridge, U.K.	C-myc oncogene product	Sikora et al., 1985

Table 3.1

Sources and antigenic determinants of the antibodies used in the immunocytochemical studies.

cases, the peroxidase end-product was developed using diaminobenzidine tetrahydrochloride/ H_2O_2 , followed by haematoxylin counter staining.

Reactions with the CAM 5.2 and NSE antibodies for each cell line studied have been graded depending on the number of cells with positive reaction and the intensity of the staining. All slides were examined by the same experienced pathologist, and graded according to the scale shown in Table 3.2.

In collaboration with Dr F. Hay (I.C.R.F., Medical Oncology Unit, Western General Hospital, Edinburgh) the cell lines were screened for cellular antigens that have previously been reported to react with a range of monoclonal antibodies (Table 3.1). Again each preparation was examined by an experienced researcher who scored the reaction according to the number of cells showing uptake and the intensity of uptake. In this case uptake has been expressed in percentage terms but the "grading" equivalents as described above (Table 3.2) are also shown.

Cytogenetic Analyses

Flasks of cells were fed with fresh medium 24 hours prior to harvesting for chromosome preparations. The harvesting protocol differed between cell lines in order to obtain optimum results. The period of exposure to 0.02% colchicine varied between 1 and 3 hours, prior to a 0.075M KCl hypotonic treatment time of 10 to 15 minutes.

Grade	Percentage of Cells Showing Uptake	Description of Staining
-ve	Nil	None
+	<5%	Modest
++	5 - 50%	Moderate
+++	>50%	Intense

Table 3.2

Grading systems used to quantify antibody staining.

Cells were then fixed in two changes of methanol:acetic acid (3:1) and stored overnight at 4°C. The next morning the fixative was changed once more and cells were dropped from varying heights onto clean, cold, wet slides. Preparations were also made from the monolayer SCLC cell line, LS112ST, by trypsinising the cells with 0.25% trypsin/EDTA solution and washing in complete medium prior to hypotonic treatment.

Fifty cells from each cell line were analysed after staining with Giemsa and/or Trypsin-Giemsa banding. The chromosome count, specific chromosomal re-arrangements and the presence of structures which may represent sites of gene amplification [i.e. homogeneous staining regions (HSRs), abnormally banding regions (ABRs) and double minute chromosomes (DMCs)] were noted. Analysis was performed at x 1280 magnification, using a Zeiss photomicroscope and photographed with Kodak Technical Pan Film.

Estimation of Dopa-decarboxylase (DDC) Activity

Principle

DDC activity is measured by the rate of conversion of ^3H -L-Dopa to ^3H -dopamine and CO_2 . ^3H -dopamine is separated from ^3H -L-dopa by liquid cation exchange.

1. Sample Preparation

The cells were centrifuged at 200g for 5 minutes and the

supernatant discarded. The cell pellet was washed twice in PBS, resuspended in 2 mls of borate buffer and lysed by freezing and thawing three times in solid CO₂ in methanol and a 37°C water bath. The lysates were centrifuged in an Eppendorf centrifuge (13,000 rpm) for 5 minutes to remove particulate material.

2. DDC Assay

The reaction mixture consisted of a borate buffer (Borax 0.025 M + KH₂PO₄ 0.05M, pH 7.0) containing nialamide (125uM), EDTA (1.5mM), L-Dopa (1200 uM) and ³H-L-Dopa (30 uCi/ml). A concentrated stock solution of the reaction mixture was prepared and 45 ul placed in 15 ml stoppered glass centrifuge tubes. These were incubated at 37°C for 5 minutes. Samples were mixed with pyridoxal-5-phosphate (1,200 uM) to give a final pyridoxal-5-phosphate concentration of 400 uM. Fifteen ul of this solution was added to the reaction mixture in the glass tubes. Samples were incubated for 1 hour at 37°C and the reaction was stopped by addition of 5 mls ice-cold sodium phosphate buffer (10 mM, pH 6.5). The tubes were transferred to ice and 1 ml of heptan-3-one containing 25 mg sodium tetraphenylboron was added. The two phases were mixed by shaking for 1 minute and then separated by centrifugation at 1500g, 4°C for 5 minutes. The lower aqueous phase was removed and the organic phase was washed with 2 mls of sodium phosphate buffer (10 mM, pH 7.4) containing 1 mg of sodium tetraphenylboron. Tubes were shaken and centrifuged at 1500 g, 4°C for 5 minutes. A sample (0.5ml) of

the organic phase was transferred to a scintillation vial and 10 mls of Ecoscint (National Diagnostics, Somerville, New Jersey, U.S.A.) added. Samples were shaken and counted for 10 minutes in a scintillation counter (Packard Liquid Scintillation Counter; Canberra Packard, Pangbourne, U.K.).

Some ^3H -L-Dopa was extracted into the organic phase and hence control tubes containing the reaction mixture but not sample were processed to estimate the reagent background counts. Extraction efficiency for ^3H -dopamine was determined by extraction of known amount of ^3H -dopamine. A sample of the reaction mixture was counted to determine the specific activity of ^3H -L-Dopa. This value was used to convert counts per minute (cpm) to μmol of dopamine. DDC activity is expressed as International Units/mg protein, where 1 I.U. = 1 μmol of dopamine formed, per minute under the specified reaction conditions.

Estimation of Creatine Kinase (CK) Activity

Principle

The principle of this assay is to measure the ability of CK to catalyze the reaction between creatine phosphate and ADP to creatine and ATP. The ATP so produced reacts with glucose in the presence of hexokinase to produce ADP and glucose-6-phosphate. This glucose-6-phosphate (G6P) is converted to 6-phosphogluconate and NADH by G6P dehydrogenase. NADH formation is measured by the

absorbance at 340 nm. The rate of change in absorbance is directly proportional to CK activity.

CK Assay

Creatine kinase (CK) reagent (Sigma Diagnostics, St. Louis, U.S.A.) is reconstituted with deionized water and mixed by inversion. This reagent not only contains creatine phosphate (the substrate for CK) but also NAD (2 mmol/l), ADP (2 mmol/l), hexokinase (yeast) (3000 u/l) and glucose-6-phosphate dehydrogenase (2000 u/l) which are all components of the chain reaction described above. The reagent also contains N-acetyl-L-cysteine (20 mmol/l) incorporated to activate the CK. One ml of the CK reagent and 0.02 ml of supernatant from the sample (prepared as above in methods for DDC estimation) were incubated at 30°C for 3 minutes. Absorbance at 340 nm was then read versus water as reference; this represents initial absorbance. The incubation was continued for a further 2 minutes and after this time absorbance was again measured (final absorbance). CK activity was then calculated from the change in absorbance at 340 nm and expressed as units of activity where one unit is defined as the amount of enzyme which produces one micromole of NADH per minute under the specified conditions.

CK Isoenzyme Analysis

Creatine kinase isoenzyme (CK-BB, CK-MM, CK-MB) profiles were determined electrophoretically. Samples were diluted such that

the activity was less than 0.7 IU/ml and 1ul was loaded onto a preformed agarose gel film (1% w/v, Corning Diagnostics Ltd., Halstead, Essex). The separating buffer was 3-(N-morpholino)-2-hydroxypropane sulphonic acid (MOPSO, 0.05M, pH 7.8) and the gel was run for 20 minutes at 9V/cm. Following electrophoresis the film was overlaid with creatine kinase substrate solution (Corning Diagnostics Ltd.) and incubated for 20 minutes at 37°C. The gel was then dried. The substrate solution was similar to that used for the estimation of CK activity and the presence of the CK isoenzymes was detected by the production of NADH. This was quantified with a scanning fluorimeter (Helena Densitometer) at an excitation wavelength of 365 nm and an emission wavelength of 460 nm. The position of the bands in the samples was compared with that of a standard preparation (3 in 1 Level 11 Electrophoresis Control, Corning Diagnostics Ltd.). The relative proportions of the 3 isoenzymes was determined by the relative area under the curve of the densitometer trace estimated as half base x height.

RESULTS

Light Microscopy

All the cell lines demonstrate typical morphological features of small cell lung cancer and grow as floating aggregates in vitro. On histological examination of sections of the SCLC aggregates nuclear chromatin was seen distributed uniformly in a stippled pattern throughout the nuclei. Nucleoli were small and indistinct. Mitoses were numerous. The majority of cells had meagre cytoplasm. Spindle and fusiform cells were also seen (Plate 3.1). In some of the larger cell aggregates central necrosis could be seen. This was particularly seen in the larger aggregates of cell line LS111.

In the two cell lines established from mouse xenografts, light microscopy revealed typical areas of small cell lung carcinoma, but also areas of bizarre, giant cells.

Electron Microscopy

The ultra-structural features of the 5 cell lines studied to date are detailed in Table 3.3. Membrane-bound dense core granules were convincingly apparent in 3 of the cell lines (Plate 3.2). In contrast, features of epithelial differentiation such as desmosomes, tonofilaments and microvillous processes were only weakly expressed, if at all, certainly indicating no significant level of squamous or glandular

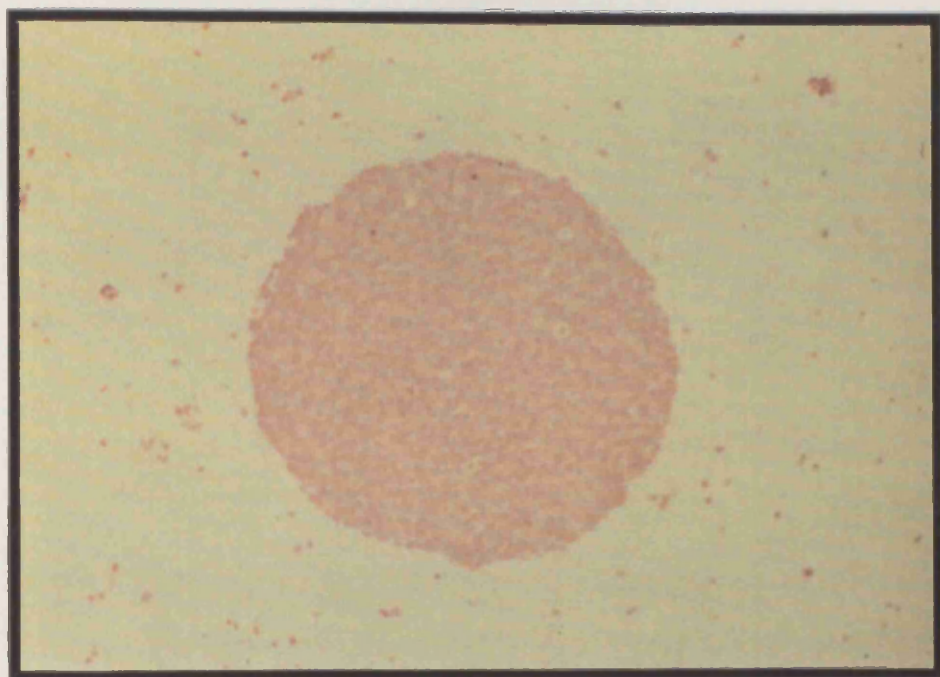


Plate 3.1

Section of LS111 tumour cell aggregate, stained with haematoxylin and eosin. In this relatively small aggregate there is no evidence of central necrosis. The characteristic features of SCLC on light microscopy can be observed.

Cell Line	Dense Core Granules	Adhesion Specialisation	Tonofilament Bundles	Microvillous Processes
LS106	-	-	+	-
LS111	++	-	-	-
LS111 Clone	+	+	-	-
LS112FL	+	+	+	-
LS112ST	-	-	-	-
LCPH2	-	+	-	-
LCPH3	+	-	-	-

+ = present but scanty.
 ++ = abundant.

Table 3.3

Ultrastructural features in SCLC cell lines.



Plate 3.2

Transmission electron micrograph prepared from cell line LS111. Membrane-bound dense core granules are clearly seen within the cytoplasm (arrowed).

differentiation in the material examined.

Immunocytochemistry

The results of the immunocytochemical studies are shown in Table 3.4. The reaction with the epithelial marker CAM 5.2 (raised against the cytokeratin cellular epitope) was most marked in cell lines LS112 and LS263 (Plate 3.3), and least marked in the small cell lines obtained from mouse xenograft and the reference cell line, NCI-H69. There was no reaction with CAM 5.2 in 3 of the 4 most recently established cell lines. On the other hand reaction with the NSE monoclonal antibody was more marked in the reference SCLC cell line and the xenograft lines, although positive staining was seen in all cell lines examined.

It may be seen that there was a spectrum of reactivity to a range of antibodies. The epithelial marker antibody HMFG2 was most strongly reactive with cell lines LS106 and LS112 (FL and ST). Again less reaction was noted with the cell lines obtained from mouse xenograft. Staining with EGFD10 was most marked in the two cell lines from mouse xenograft. Bombesin reactivity was most marked in cell line LS111. The reference cell line (NCI-H69) and LS106 showed the weakest reaction with bombesin. Studies using the monoclonal antibody raised against the C-myc oncogene were negative in 5 of the 8 new SCLC cell lines tested and weakly positive with LS112ST, LS263 and LS277, as well as the reference cell line NCI-H69.

Cell Line	H69	LS103	LS111	LS111 Cbne	LS112 FL	LS112 BT	LOPH2	LOPH2	LS923	LS274	LS277	LS310
Antibody												
CAM 5.2	+	++	++	+	+++	++	+	+	+++	-ve	-ve	-ve
NSE	+++	+	+++	++	+	++	+++	++	+++	+++	+++	+++
HMF2	++ (20%)	+++ (50%)	-ve	+	++ (30%)	+++ (80%)	+	+	-ve	-ve	-ve	-ve
EGFD10	+	unsatis.	+	+	-ve	+	++ (10-20%)	++ (15%)	-ve	-ve	-ve	-ve
Bombesin	++ (10%)	+	+++ (90%)	+++ (80%)	++ (40%)	++ (20%)	++ (30-50%)	unsatis.	++ (30%)	++ (30%)	++ (30%)	++ (30%)
Anti-SCLC (123C3)	-ve	unsatis.	-ve	-ve	-ve	+	+	+++ (80%)	+	-ve	-ve	-ve
C-myc	+	-ve	-ve	*ND	-ve	+	*ND	-ve	+	-ve	+	-ve
	(1%)					(5%) strong			(10%)		(5-10%)	

*ND = not done

Table 3.4

Reactivity of a panel of antibodies with the new SCLC cell lines, and for comparison, the continuous SCLC cell line

NCI-H69. Grading of reactivity is as specified in Table 3.2.

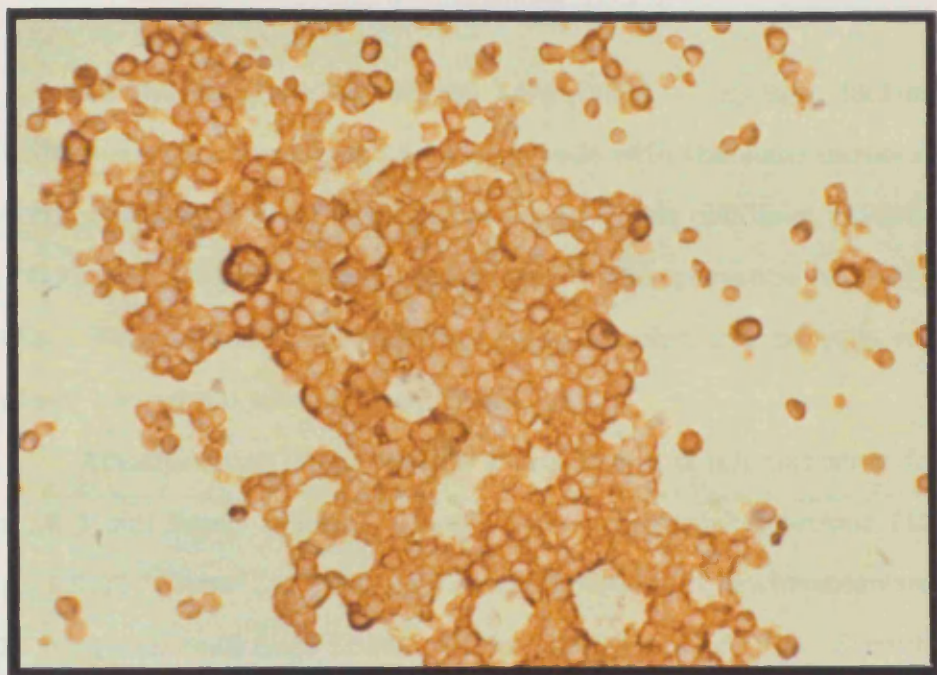


Plate 3.3

A paraffin section of cell line LS263 stained with the antibody CAM 5.2 directed against an epithelial antigen. Strong positive staining is observed (+++, 90%).

Cytogenetic Analyses

All the cell lines had human karyotypes. Cell lines had modal chromosome concentrations (5 or more cells with the same chromosome count) ranging from 39 to 53 chromosomes. Two cell lines (LS106 and LS277) had a bimodal distribution due to the presence of polyploid cells. Karyotypes of all cell lines were complex and no cells with a normal karyotype were found (Table 3.5).

Abnormalities of chromosome 3 (short arm deletions) were found in all 9 cell lines. These included both interstitial deletions (LS111 and LS111 "Clone", LCPH2, LCPH3 and LS263) and isochromosomes for the long arm (cell lines LS106, LS274, LS277 and LS310). Structures which may contain amplified gene sequences (HSRs, ABRs, and DMCs) were found in 3 cell lines [LS112 (FL and ST), LS263 and LS277]. Cell line pairs (LS111 and LS111 "Clone", LS112FL and LS112ST, LCPH2 and LCPH3, LS274 and LS310) showed only minor karyotypic differences. The karyotype of LS111 is shown in Plate 3.4, which demonstrates the characteristic 3p deletion.

Measurement of Enzyme Activities

The results of the enzyme assays for L-dopa-decarboxylase (DDC) and for creatine kinase (CK) and its isoenzyme profile are shown in Table 3.6. All the newly established small cell lung cancer cell lines exhibit activity of the SCLC marker enzyme DDC with a wide

Cell Line	Loss or Gain of Normal Chromosomes	Structural Aberrations
LS106	-1,-3,-4,+8,-9,-10,-13, -15,+18,-21,-22	
LS111	-3,-5,-5,-10,-13,+21	
LS111 (Clone)	-3,-5,-5,-10,-13,+21	
LS112 FL	-1,-2,-3,-4,-5,-6,-7,+8, -10,-12,-13,-14,-15,-15, -16,-17,-17,-18,-19,-19,-21	*HSR (Chromosomes 11 + 12) + 8 markers
LS112 ST	-1,-1,-2,-3,-4,-5,-5,-7,+8, -10,-11,-12,-13,-14,-15,-16, -17,-17,-18,-19,+20,-21,-22,-X	HSR (Chromosomes 11 +12) +6 markers
LCPH2	-3,-4,-5,-6,+7,+14,+18,+X	
LCPH3	-3,-4,-5,-6,+7,+13,+14,+18,+X	
LS263	-3,-4,-4,-5,-6,-7,-8,-9,-12, -13,-13,-14,-14,-16,-18,-19,-20,-21	HSR (Chromosomes 14+20) **ABR (Chromosomes 11 + 14) +2 markers
LS274	-3,-3,-6,-11,-12,-13,-13,-14,-17, -18,-18,-19	
LS277	-3,-4,-5,-10,-15	***DMC + 2 markers
LS310	+1,-3,-3,-11,-12,-13,-13,-14, -15,-18,-19,-22	

- * Homogeneously staining region (HSR)
- ** Abnormally bonding region (ABR)
- *** Double minute chromosome (DMC)

Table 3.5

Chromosomal characterisation of SCLC cell lines.

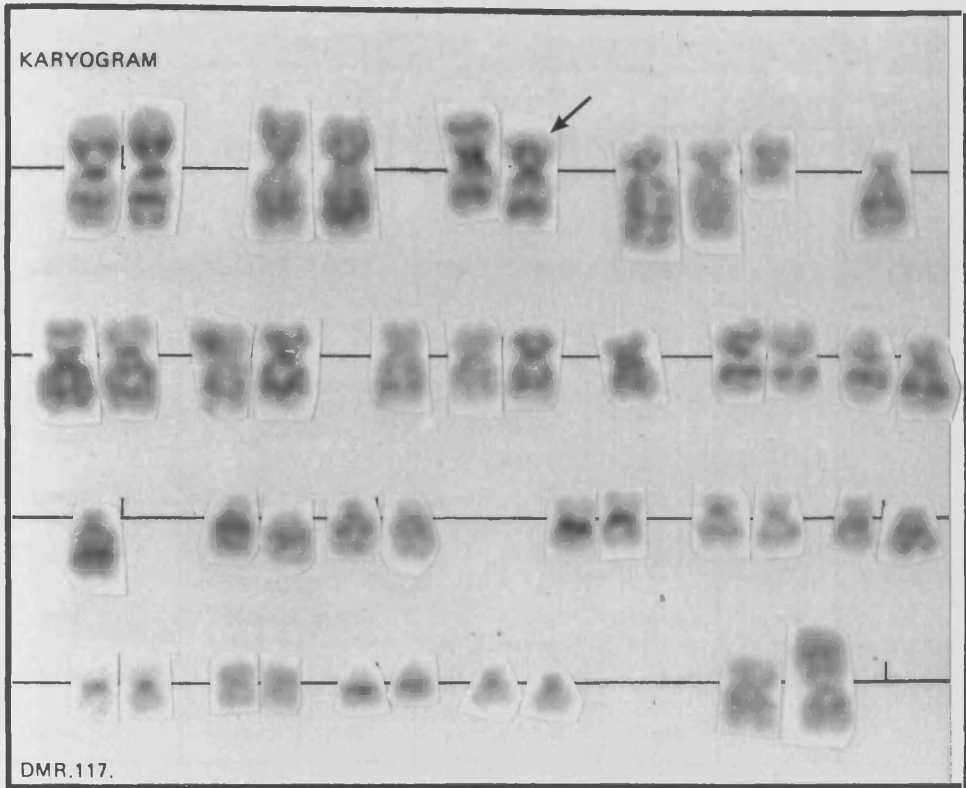


Plate 3.4

Karyotype of a representative metaphase of cell line LS111. The characteristic deletion of the short arm of chromosome 3 is arrowed.

Cell Line	DDC uIU/mg protein	CK u/mg protein	CK isoenzymes (%)		
			BB	MB	MM
LS106	66,311.9* \pm 902.2	8.4 \pm 1.1	93.5	0.0	6.5
LS111	1,550.9 \pm 197.8	5.0 \pm 0.8	100.0	0.0	0.0
LS111 Clone	2,272.7 (n=2)	5.9 (n=2)	100.0	0.0	0.0
LS112 FL	2,220.8 \pm 773.9	0.4 \pm 0.2	54.6	0.0	45.4
LS112 ST	2,857.4 \pm 1372.5	0.4 \pm 0.1	40.9	0.0	59.1
LCPH2	N.D.	N.D.	-	-	-
LCPH3	2,224.6 \pm 185.7	10.0 \pm 2.2	97.7	0.0	2.3
LS263	1,117.3 \pm 206.8	0.78 \pm 0.1	92.2	0.0	7.8
LS274	14,275.4 \pm 2012.5	4.5 \pm 1.1	73.6	19.9	6.5
LS277	450.4 \pm 4.1	0.8 \pm 0.1	79.4	8.2	12.4
LS310	11,796.3 \pm 1703.6	5.1 \pm 1.0	89.5	7.0	3.5

N.D. = not done.

Values are mean \pm SEM of 4 observations.

Table 3.6

Activities of the marker enzymes Dopa-decarboxylase (DDC) and Creatine Kinase

(CK) in newly established SCLC cell lines.

range in activity. In the case of creatine kinase, activity is similar for all cell lines except for LS112 (both FL and ST), LS263 and LS277 where levels of activity are much lower, although still present in significant quantity. With regard to the CK isoenzyme profile all cell lines expressed the BB isoenzyme. This was the dominant form except for cell line LS112 (FL and ST) where there were equal proportions of the MM and BB isoenzymes. It is also of note that the MB isoenzyme was present in 3 of the cell lines (LS274, LS277 and LS310).

There did not appear to be any specific relationship between DDC and CK enzyme activities in the new SCLC cell lines. For example, LS112 with low CK activity had intermediate levels of DDC activity whereas LCPH3 with the highest levels of CK activity also had intermediate levels of DDC activity.

DISCUSSION

These results show that the established cell lines are of human small cell lung cancer origin in all cases. This has been shown to be the case not only on pathological (light, ultrastructural and immunocytochemical) examination and on karyotypic analysis but also by the activity of the small cell marker enzyme CK-BB (Gazdar et al., 1985). All the above characteristics have been well described by previous workers as detailed in the introduction of this Chapter, as has the spectrum of characteristics seen in newly established SCLC cell lines.

On the basis of morphological appearance and biochemical profile the 9 cell lines which have been established demonstrate a range of biological characteristics ranging from classical (LS111) to variant (LCPH3) but with a number of cell lines that fall into an intermediate category. As can be seen from Table 3.7, each characteristic has been divided into categories. Although these divisions are quite arbitrary it has allowed a satisfactory categorisation of these newly established SCLC cell lines (Table 3.8). It may be seen that each cell line did not show a homogeneous pattern of classic or variant characteristics, unlike the work described by Carney et al., (1985A). However this categorisation did allow identification of predominantly classic (LS111), intermediate (LS106, LS112) and variant (LS263) cell lines. Ultimately it was intended to study each specific phenotypic characteristic of each cell line individually to assess if there was any

Features	Classic (C)	Intermediate (I)	Variant (V)
Morphology	Tight aggregate	Intermediate aggregates	Loose aggregates
Dopa-decarboxylase (ulU/mg protein)	> 10,000	1,500 - 9,999	< 1,500
Bombesin-like Immunoreactivity (BLI)	+++ (>50%)	++ (10-50%)	+ (<10%)
Neurone Specific Enolase (NSE)	+++ (>50%)	++ (10-50%)	+ (<10%)

Table 3.7

Parameters used to categorise SCLC cell lines into Classic, Intermediate and Variant sub-groups.

Cell Line	Morphology	Enzyme Activity (ODC)	Immunocytochemical Features		Summary
			BU	NBE	
LS108	I/C	C	V	V	I
LS111	C	I	C	C	C
LS111 Clone	C	I	C	I	I/C
LS112 FL	I	I	I	V	I
LS112 ST	V	I	I	I	I
LCPH2	I	*ND	I	C	I
LCPH3	V	I	-	I	I
LS263	V	V	I	C	I/V
LS274	V	C	I	C	I/C
LS277	I	V	I	C	I
LS310	V	C	I	C	I/C

* ND = not done.

Table 3.8

Classification of the newly established SCLC cell lines in terms of the parameters defined in Table 3.7

The cell lines are divided into classic (C), intermediate (I) and variant (V) phenotypes on the basis of morphology, enzyme activity and immunocytochemical features. The final column summarises the overall predominant phenotype of the cell line.

relationship to chemosensitivity.

Light Microscopy

Light microscopy of sectioned tumour cell aggregates confirmed appearances of human small cell lung cancer. In the two cell lines established from mouse xenograft bizarre large cells were also present, and these probably represent transformed residual mouse stromal cells. Indeed, previous workers (Gazdar et al., 1980) have also reported that successful replication of cell lines derived from tumours in athymic nude mice may be dependent on the presence of mouse stromal cells for periods of up to 24 months.

Electron Microscopy

The ultrastructural features seen in SCLC cell lines have already been described in the Introduction. In summary, although dense core granules (DCGs) have been described as characteristic more recent work has shown that they are not always present (Carney et al., 1985A; De Leij et al., 1985) and features of epithelial differentiation (De Leij et al., 1985; Iglehart et al., 1985) and adenodifferentiation (Mooi et al., 1986) have also been found. Indeed the difficulty of always finding DCGs in SCLC has recently been highlighted (Dunnill & Gatter, 1986).

Clear evidence of dense core granules were found in the most

classic SCLC cell line, LS111 (Plate 3.2). There was evidence of DCGs in 2 of the other 4 cell lines examined. Thus DCGs were not identified in the preparations examined from 2 of the cell lines (LS106 and LCPH2). This is not entirely unexpected, particularly as these two cell lines fall into an intermediate/variant classification and the absence of DCGs in variant SCLC cell lines has been previously described (De Leij et al., 1985). There were also features of epithelial differentiation and adenodifferentiation in 3 and 2 of the cell lines respectively (Table 3.3).

Immunocytochemistry

The immunocytochemical studies undertaken have been useful in confirming the epithelial features of the newly established cell lines with varying positivity with CAM 5.2 and HMFG2 noted. Such a spectrum of positivity to markers of epithelial differentiation has been noted previously, as described in the Introduction to this Chapter. In addition a spectrum of immunoreactivity was seen with antibodies directed against the SCLC marker enzymes bombesin and neurone specific enolase (NSE). Using the antibodies raised against bombesin and against NSE along with biochemical estimation of dopa-decarboxylase it has been possible to classify the cell lines into classic, intermediate and variant categories. In general terms there was a good correlation between those cell lines with classical biochemical profiles and those with classical (or intermediate)

immunocytochemistry. However cell line LS106 was an exception to this rule, with high DDC activity but low NSE and bombesin-like immunoreactivity (BLI). In addition cell lines LS263 and LS277 had low levels of DDC, and intermediate BLI but high levels of NSE activity on immunocytochemical testing. Similarly there was an association between cell lines with variant biochemical profiles and with variant (or intermediate) immunocytochemistry. Thus these immunocytochemical studies have not only proved useful in characterizing the cell lines but also in corroborating the results of biochemical analyses.

Studies using recently raised antibodies also proved useful in the further characterisation of the cell lines. HMFG2 appeared in general to be similar in reactivity to CAM 5.2 except for cell lines LS111 and LS263. This is what one might expect given that both are epithelial markers. In contrast, EGFD10 generally showed only weak activity, if any, and did not appear to mirror CAM 5.2 and HMFG2 activities. The anti-SCLC marker (123C3) probably directed against SCLC neurofilaments was only strongly positive in LCPH3, which was the most variant of all the cell lines. This finding would corroborate those of Broers et al., (1985) who suggested that variant SCLC cell lines usually have neurofilaments. Three of the newly established cell lines showed a weak positive reaction with the C-myc antibody. The significance of this remains uncertain.

Cytogenetic Analyses

The various cytogenetic abnormalities reported in SCLC have been described in detail in the Introduction to this Chapter. Abnormalities of the short arm of chromosome 3 were found in all 11 cell lines and sub-lines (LS111 and LS111 "Clone"; LS112FL and LS112ST) studied. In 9 of these (6 patients) the characteristic 3p deletion (Whang-Peng et al., 1982) was detected. A host of additional chromosomal defects were found with chromosome numbers ranging from 39 to 53 and over-representation of chromosome X and extra material on chromosome Y (Table 3.5 and Plate 3.4). There was an under-representation of chromosome 13 in 6 cell lines from 4 patients. Such an under-representation presumably includes not only chromosome loss but structural rearrangements of chromosome 13 that are masked within the complexity of the karyotype and both could be a mechanism for the loss of alleles. In all published cases where detailed karyotypic data on SCLC is given, 37 of 63 cell lines (59%) have shown an under-representation of chromosome 13.

Considering the 9 cell lines analysed, there were 4 cell line pairs in which each pair had been established from the same patient (LS111 and LS111 "Clone", LS112FL and LS112ST, LCPH2 and LCPH3, and finally LS274 and LS310). Of these, the first two pairs had a common tumour origin and the latter two were established from tumour biopsies from the same patient at two stages of their disease. Very few karyotypic alterations between each member of a pair were

apparent even though they had been separated for many months in tissue culture. Not only did they exhibit karyotypic stability, but even separate tumours from the same patient (LCPH2 and LCPH3, LS274 and LS310) had a large proportion of their chromosome aberrations in common, showing that these had probably arisen in vivo, during tumour formation and were not artefacts of tissue culture. The pair of cell lines LS112FL and LS112ST have different growth morphologies and it is interesting to speculate that these differences could result from the additional 3 marker chromosome found in the monolayer culture, LS112ST.

In summary, it has been confirmed that all the cell lines are of human tumour origin. The karyotypic data obtained from these cell lines confirms the consistent deletion of material from the short arm of chromosome 3 and suggests a possible role for this and the loss of material from chromosome 13 in tumour progression in SCLC.

Biochemical Studies

The biochemical analyses proved useful in confirming the nature of the origin of the cell lines, with all demonstrating significant activity of the SCLC marker enzyme CK-BB. Indeed in the majority of cases, high levels of CK-BB activity were found. A number of cell lines demonstrated CK-MM, as well as CK-BB activity. This has previously been reported (De Leij et al., 1985; Postmus et al., 1988) but the significance of this finding remains uncertain.

The spectrum of activity of L-dopa-decarboxylase proved useful in categorising the cell lines into classic, intermediate and variant. As discussed above this characteristic showed good general agreement with the results of immunocytochemical studies.

Summary

Nine small cell lung cancer cell lines have been established and characterised. Pathological studies, including light and electron microscopy and immunocytochemistry, and cytogenetic analyses, as well as biochemical analyses have clearly demonstrated that these lines are of human small cell lung cancer origin. A spectrum of characteristics, ranging from classic to variant, with the majority of cell lines having intermediate features have been found.

These cell lines form the basis of the studies of drug sensitivity and modulation in vitro. However, the SCLC cell lines all grow as floating aggregates in vitro and this resulted in difficulties in studying their growth and chemosensitivity. The problems encountered and the means developed to overcome them are discussed in the next section of this thesis.

CHAPTER 4

ESTIMATION OF GROWTH RATE AND CHEMOSENSITIVITY BY AGGREGATE GROWTH AND GROWTH DELAY ANALYSIS

INTRODUCTION

A major aim of this project was to establish new small cell lung cancer cell lines in the laboratory to use as models for studies of drug resistance. Thus having successfully established and characterised 9 SCLC cell lines in vitro, an assay for measuring drug sensitivity in these cell lines was required. Because of the large number of cell lines established and in view of the fact that ultimately it was intended to assess a range of cytotoxic drugs, the assay chosen for measuring growth and chemosensitivity had to be relatively simple, reproducible and sensitive enough to be capable of detecting small changes in drug sensitivity. This latter point was particularly important as the model system established would also be used to assess the activity of a number of resistance modifying agents.

All the newly established small cell lung cancer cell lines described in the preceding Chapters grow as floating aggregates in suspension. This is a characteristic of almost all SCLC cell lines that grow continuously in vitro (Carney et al., 1985A; Gadzar et al., 1980). This very fact however poses major problems in the study of these lines in the laboratory, for it renders them unsuitable for

assessment of both growth and chemosensitivity using conventional assays.

Clonogenic Assays

The majority of workers in this field have attempted to study growth and hence chemosensitivity in both fresh biopsies and in established cell lines using a form of clonogenic assay (Carney et al., 1980, 1983; Hamburger & Salmon, 1977; Hamburger et al., 1978; Kahn et al., 1986; Sarosdy et al., 1986). Gazdar and associates (1985) from the National Cancer Institute popularised the use of this system in SCLC. However, they themselves have highlighted the problem of obtaining a satisfactory single cell suspension from floating aggregates (Carney et al, 1983). Indeed the problem of clumping in studies of both fresh biopsies (Alley & Lieber, 1984; Bertoncello et al., 1982; Hamburger et al., 1982; Twentyman, 1985) and in cell lines (Ruckdeschel et al., 1987; Walls & Twentyman, 1985) has never been satisfactorily overcome. Recently Ruckdeschel et al., (1987) demonstrated significant cell death when trying to obtain a single cell suspension from SCLC cell lines. Furthermore, even when the technical problems are minimised, such assays are limited by the poor colony forming efficiency of lung cancer cells in vitro (Gazdar et al., 1983; Salmon & Von Hoff, 1981; Von Hoff et al., 1981). In any case it seemed more appropriate to study the intact aggregate as this is more analogous to the in vivo situation (Plate 4.1).

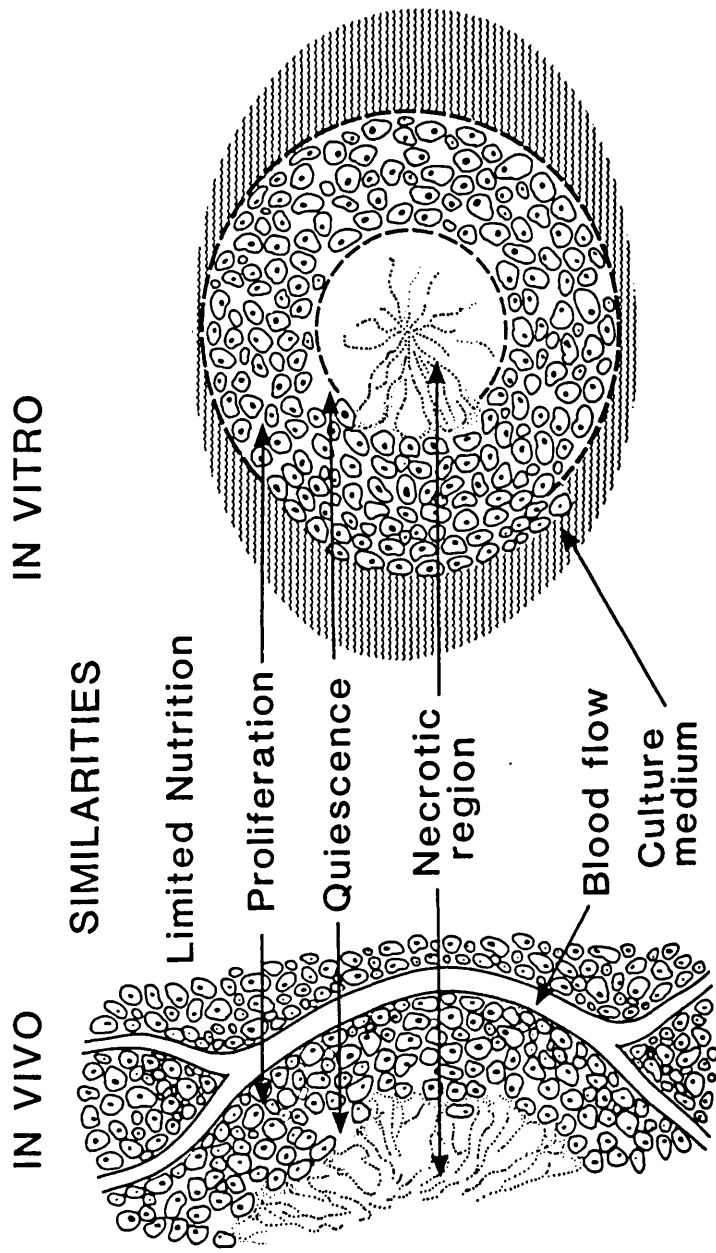


Plate 4.1

This illustration emphasises the similarity between multicellular tumour spheroids in vitro and tumour growing in vivo.

Moreover the relevance of the clonogenic assay has been questioned (Weisenthal & Lippman, 1985). In particular true tumour stem cells may be non-dividing (Go) cells and therefore clonogenic cells may not represent tumour stem cells. Indeed growth kinetic studies have suggested that tumours in vivo are made up of a mixture of dividing cells and temporarily non-dividing (Go) cells (Gabutti et al., 1969; Saunders et al., 1969). In addition Suda et al. (1983), in studies of normal murine haematopoiesis, have shown that stem cells do have long variable Go periods, compared to cells committed to terminal differentiation, with purely random activation of stem cells from this population. Suda et al. (1983) went on to show that reversibly non-dividing Go cells may not be assayed using a clonogenic system and re-iterated that these are probably true stem cells. Moreover it has been shown that cells which are committed to differentiation can form colonies in vitro (Mackillop et al., 1983).

It has been postulated that it may be the failure to kill Go cells that is partly responsible for the ineffectiveness of chemotherapy in human cancers (Drewinko et al., 1981). Certainly concerns about the clonogenic assay in terms of clinical credibility have often been expressed. Not only because of the artificial environment and abbreviated and arbitrary drug exposure (Selby et al., 1983) which are indeed criticism of any in vitro assay, but also because by using a clonogenic assay to predict for survival one ignores the problem of resistant tumour sub-populations (Skipper & Schabel, 1984; Stephens et al., 1984; Tofilon et al., 1984).

For these reasons it was felt that an alternative approach to studying growth and chemosensitivity in the SCLC cell lines was needed, and the various possible non-clonogenic assays are briefly reviewed here to indicate why the system selected was chosen.

Alternative Techniques for Measuring Growth and Chemosensitivity

By far the simplest way to measure growth is to count increasing cell number over a period of time. This can most easily be performed by counting single cells obtained after trypsinisation in a coulter counter. However, for reasons stated above it seemed likely that the cell line aggregates might prove difficult to disperse into true single cell suspension.

An alternative method to assess growth might be to perform protein assays over time (Bradford, 1976; Lowry et al., 1951). Such assays however measure dead as well as live cells. Moreover protein production is not a constant. Measurement of total DNA (Labarca & Paigen, 1980) is another technique which has been used to estimate cell growth. However, again this assay would measure DNA in dead as well as live cells. Furthermore the amount of DNA per tumour cell is not constant. Hence neither protein assays nor DNA assays seemed appropriate.

Proliferation Assays

Assays which measure the uptake of a radioactive labelled precursor are often used to measure chemosensitivity. These assays are based on the premise that living but not dead cells will take up radioactive labelled precursors (Volm et al., 1979). Specific examples include tritiated thymidine or leucine (Morgan et al., 1983). Over the last decade a number of workers have reported the usefulness of measuring tritiated nucleotide incorporation to measure chemosensitivity (Ballou & Tseng, 1986; Sanfillipo et al., 1986; Silvestrini et al., 1985). However, tritiated thymidine is only taken up by some cells in tissue culture and then only by cells in S-phase. Although tritiated leucine is taken up by all cells it effectively measures protein turnover, which will be variable depending on the stage of the cell cycle. Furthermore, techniques involved in assessing growth using tritiated leucine uptake are labour intensive and time consuming. In addition the time of performing the labelling assay after the noxious insult is critical. If nucleoside label incorporation is estimated too early then specific problems will occur. This relates to artefactual alterations in nucleoside intracellular pool sizes (Nakata & Bader, 1969). In addition the problem of salvage versus de novo dTMP synthesis has to be considered (Wolberg, 1972).

It has been shown that some cells have a long cycle time (Livingston et al., 1980). Indeed Raich (1978) demonstrated that acute myelogenous leukaemia blasts may have a cycle time of up to 70

hours. Furthermore it has been shown that many samples of tumour do not incorporate sufficient amounts of ^3H -thymidine to allow accurate measurement and comparisons (Bech-Hansen et al., 1976, 1977). In addition Hamburger (1981) has pointed out that precursor incorporation into cells may be temporarily stopped due to damage to cells during the period of handling in the experiment. She also re-emphasized the profound effects of anti-cancer drugs on nucleotide synthesis which can therefore artefactually decrease estimates of DNA synthesis.

A final problem often faced by those using labelled precursor uptake studies to measure tumour growth and chemosensitivity is that of differentiating between tumour and non-tumour uptake. Some researchers have applied autoradiographic techniques to overcome this difficulty (Livingston et al., 1980; Sky-Peck, 1971; Weisenthal et al., 1984). Although this latter difficulty is not a specific problem in the new SCLC cell lines, it does underline the importance of critical consideration before an appropriate assay is finally chosen. Thus these techniques did not seem suitable for estimating the chemosensitivities of the SCLC cell lines.

Metabolic Assays

The possibility of measuring growth and chemosensitivity using changes in cell metabolism as an end-point was considered. Changes

in oxygen consumption (Laszlo et al., 1958) and measurement of ^{14}C labelled CO_2 released into the gas phase of culture bottles containing ^{14}C labelled glucose (Scheithauer et al., 1986) are very sensitive to culture conditions and therefore difficult to control.

Assays of the Entire Cell Population

An alternative approach is to look for gross cell damage in the entire population in morphological terms. Weisenthal & Lippman (1985) have suggested that very gross early evidence of cell damage in the whole tumour may correlate with a multiple log cell-kill in that population of tumour cells which is responsible for sustained tumour growth. Studies of tumour growth in vivo as xenografts (Fergusson et al., 1986) or using a sub-renal capsule assay (Bogden et al., 1984; Bogden & Van Hoff, 1984) would achieve this objective, although the importance of histological examination for tumour cell damage (and not simply tumour size) has been emphasized (Dumont et al., 1984; Levi et al., 1984). Such techniques are expensive, labour-intensive (especially when histological tumour examination is performed), require the presence of an animal house facility and most importantly take a longer period of time to obtain meaningful results.

Cell Membrane Function Assays

Assays based on changes in cell membrane function have also

been used to study chemosensitivity. This approach assesses damage to cells by the inability of injured cells to exclude a dye as first described by Kaltenbach et al. (1958). Subsequently a number of dye exclusion assays, using eosin Y, erythrocyin B, nigrosin and trypan blue, with or without internal standards, have been described (Durkin et al., 1978; Roper & Drewinko, 1976,1979; Tsukeda et al., 1978; Weisenthal et al., 1983A,B). An alternative is to estimate injury by the ability of viable cells to retain a dye such as fluorescein (Rotman & Papermaster, 1966). Differential staining assays of cell suspensions (Bird et al., 1986; Weisenthal et al., 1983B) and of fixed cells (Kornblith & Szytko, 1978; Leu & Herriott, 1984; Mealey et al., 1974) have also been described.

In recent years there has been a rekindling of interest in this technique (Weisenthal et al., 1983B). Such assays are quick and relatively simple and require less cells than cloning assays. Furthermore they detect cell-kill in the Go fraction of cells. Most important perhaps is that such dye assays do not require a strict single cell suspension. However, there are problems as recently reviewed by Weisenthal & Lippman (1985). For example, whilst allowing sufficient time for damaged membranes to lose their integrity, the surviving cells may continue to proliferate (Hamburger, 1981). Most importantly however is the labour intensive nature of scoring damaged cells (Ruckdeschel et al., 1987). Given the large number of cell lines to be studied and the range of cytotoxic drugs and resistance modifiers to be screened, a simple, quick assay that could

be semi-automated was required.

A number of other short term assays of drug-induced injury have also been described including those based on dehydrogenase activity after insult (Di Paolo, 1965) and more recently the sister chromatid exchange assay (Tofilon et al., 1984) has been shown to be able to detect small changes in drug induced cytotoxicity. This latter assay is rather complex and again depends on analysis of individual cells.

Spheroid Growth Studies

One of the more promising approaches used previously was based on changes in the measurement of the total tumour cell population in vitro. For example, Berry et al. (1975) examined changes during the continuous short term exposure of mono-layer cultures exposed to cytotoxic drug. Specifically with regard to the SCLC aggregates, in the last decade there has been a great increase in interest in measuring changes in multicellular tumour aggregates (or spheroids) both to measure growth per se and to quantify toxic injury by measuring the growth delay induced by such insult. This latter approach seemed to be particularly promising and from the literature it seemed feasible to study the newly established SCLC aggregates in the sort of system that had previously been described (Sutherland & Durand, 1984; Yuhas et al., 1978). Such

measurements can be performed over a period of time using a televisual system interlinked to a computer. The computer can be used to convert area measurements to volume (Wheldon et al., 1987). This system has the advantage that effects of drug on defined growth patterns can also be measured (Nederman & Twentyman, 1984), while preserving cell-cell contact and interactions (Miller et al., 1984; Weisenthal & Lippman, 1985).

It seemed likely that spheroid growth studies to define normal growth and to determine the effects of cytotoxic damage might be a valid method to study growth and chemosensitivity in the new SCLC cell lines. Such an approach has been previously described in SCLC (Douple et al., 1985). Moreover this approach avoids the need to obtain single cell suspensions thus avoiding cell injury from disruption of the aggregates and so allowing the maintenance of cell-cell interactions within the spheroid during cytotoxic drug exposure. Clearly this is more analagous to the in vivo situation (Miller et al., 1984). Since the necessary apparatus and expertise was readily available spheroid growth studies were assessed for their application to the measurement of growth and chemosensitivity of the recently established small cell lung cancer cell line aggregates.

METHODS

Growth Studies

Small spheroids (approximately 50-100 microns in diameter) were transferred under microscopic examination to individual wells of a 24 well test plate (Linbro; Flow Laboratories, Rickmansworth, U.K.) using glass Pasteur pipettes. The individual wells were agar base-coated with 0.5 ml of 1.25% agar made up with RPMI 2.5% SIT.

Spheroid size was monitored twice weekly by measurement of cross-sectional areas of individual spheroids using a micromeritics image analysis system coupled via a television camera to an inverted optical microscope (Wheldon et al., 1987) (Plate 4.2). These area measurements were subsequently converted to volumes assuming spherical geometry and graphs constructed of median log volume growth of spheroid against time.

Cytotoxicity Assays

Exponentially growing tumour cell aggregates in 25 mls of medium from 75cm² flasks were sub-divided into six 25 cm² flasks, each made up to 10 mls with medium. The following day the medium was carefully removed and medium containing drug at various concentrations added. For these experiments the cytotoxic drug used, doxorubicin (Farmitalia Carlo Erba, St Albans, Herts.) was



Plate 4.2

The image analysis apparatus used to measure aggregate cross-sectional area. Individual tumour cell aggregates contained in the wells of a 24-well plate are viewed under phase contrast with an inverted microscope. A television camera linked to the microscope eye-piece relays the image to the screen of the image analysis system. The surface area of the aggregate image is used to estimate volume, assuming spherical geometry.

freshly prepared and diluted using fresh medium (RPMI 2.5% SIT) to obtain a final concentration range of doxorubicin of 1.6×10^{-9} M to 2×10^{-7} M. Aggregates were exposed to doxorubicin for 24 hours at 37°C . The drug containing medium was removed and the aggregates were then carefully washed once with fresh medium at room temperature to remove residual drug. Individual spheroids were then transferred into agar-coated 24-well plates and studied as described above. For each drug concentration in each experiment at least 12 individual aggregates were studied. Graphs of median log volume against time were obtained for each concentration of doxorubicin.

RESULTS

Growth Studies

The first 5 of the newly established SCLC cell lines were studied using this system (LS106, LS111, LS112FL, LCPH2 and LCPH3). However, only 3 of the 5 cell lines studied were found to grow satisfactorily when plated out as individual aggregates in 24 well plates. These comprised cell lines of either classical (LS111) or intermediate (LS106 and LS112FL) morphology. The growth curves are shown in Figures 4.1 and 4.2. It can be seen that the growth curve for the cell line with the most classic (i.e. spheroidal) morphology had by far the tightest 95% confidence limits (Figure 4.1A). For cell lines with intermediate or variant morphology errors in volume measurements were encountered due to the non-spheroidal geometry of the aggregates (for example LS112FL, Figure 4.1B). It may also be seen that the slope of the growth curves remains similar in cell line LS106 in which growth studies were repeated after an interval of 4 months (Figure 4.2A).

The cell lines with variant morphology (LCPH2 and LCPH3) could not be studied using this system. LCPH2 did not grow at all when plated out individually, perhaps an indication of its relatively young age in tissue culture when studied. LCPH3 did show some growth, but this followed a protracted lag phase of 10 days (Figure 4.2B). The estimated volume doubling times for the 3 cell lines that

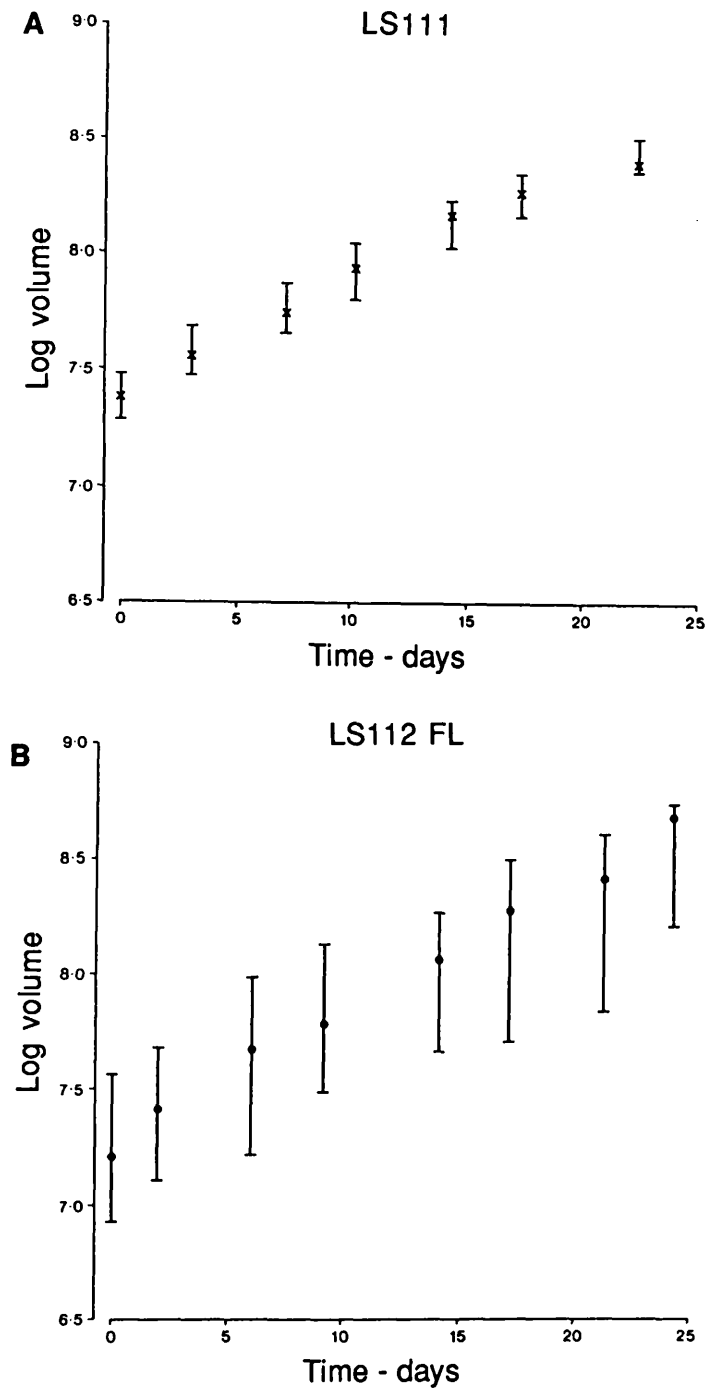


Figure 4.1

Aggregate volumes estimated at various times after plating out single aggregates into each well of a 24-well plate. Volume of the aggregate was estimated by a computerised tele-visual analysis system and values are the median \pm 95% confidence limits of 24 aggregates. Results are shown for cell lines LS111 (A) and LS112FL (B).

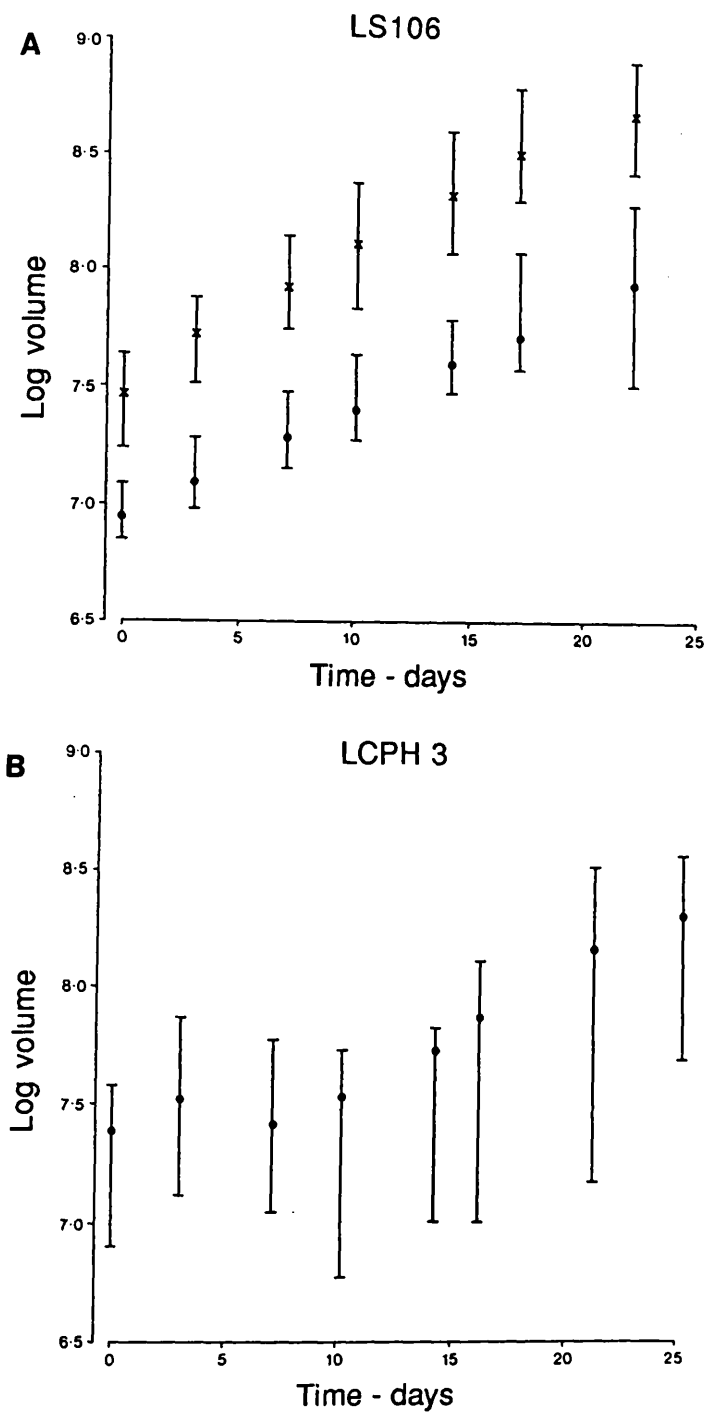


Figure 4.2

Aggregate volumes estimated at various times after plating out individual aggregates of cell lines LS106 (A) and LCPH3 (B) into each well of a 24-well plate. Values are the median \pm 95% confidence limits. For cell line LS106 (A) results of two separate experiments are shown.

did grow satisfactorily in this system are shown in Table 4.1.

Cytotoxicity Assays

Despite the limited number of cell lines that did grow in this system a number of cytotoxic drug exposure experiments were performed, principally with cell lines LS111 and LS112FL. Two representative experimental results are shown in Figure 4.3. At low drug concentrations ($1.6 \times 10^{-9}\text{M}$) little growth delay was seen, except in the case of individual small aggregates (Figure 4.3A). At intermediate drug concentrations ($8 \times 10^{-9}\text{M}$ and $4 \times 10^{-8}\text{M}$) a distinctive growth delay pattern was seen (Figure 4.3A, B). At the top drug concentration the tumour aggregates died completely and disintegrated by Day 10 (Figure 4.3A). The growth delays produced in each cell line for each cytotoxic drug concentration are shown in Table 4.1.

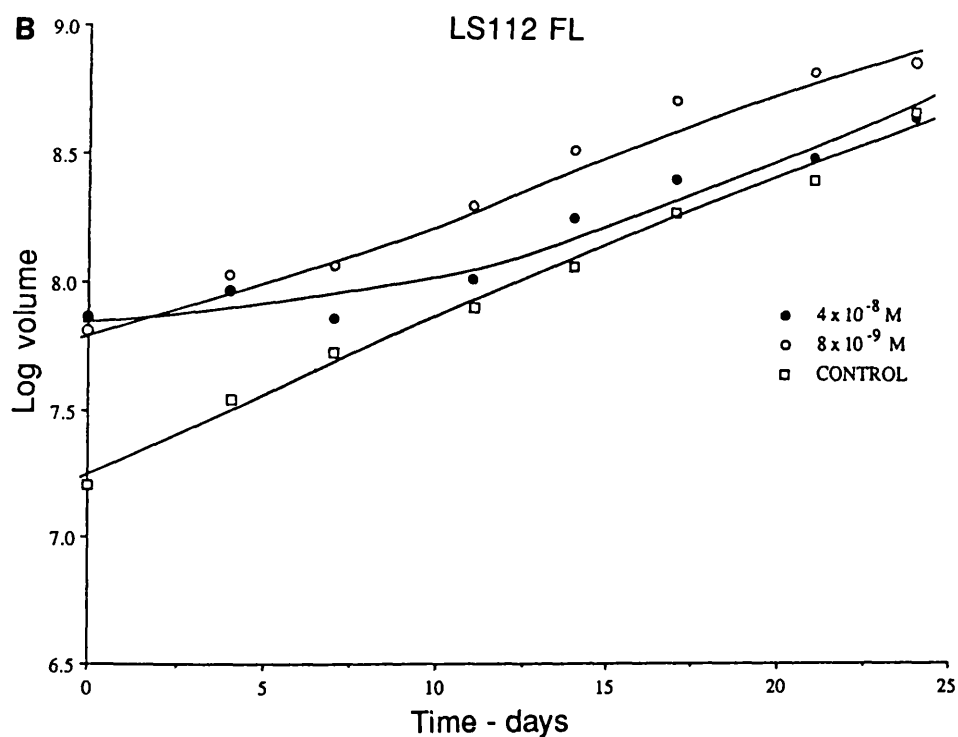
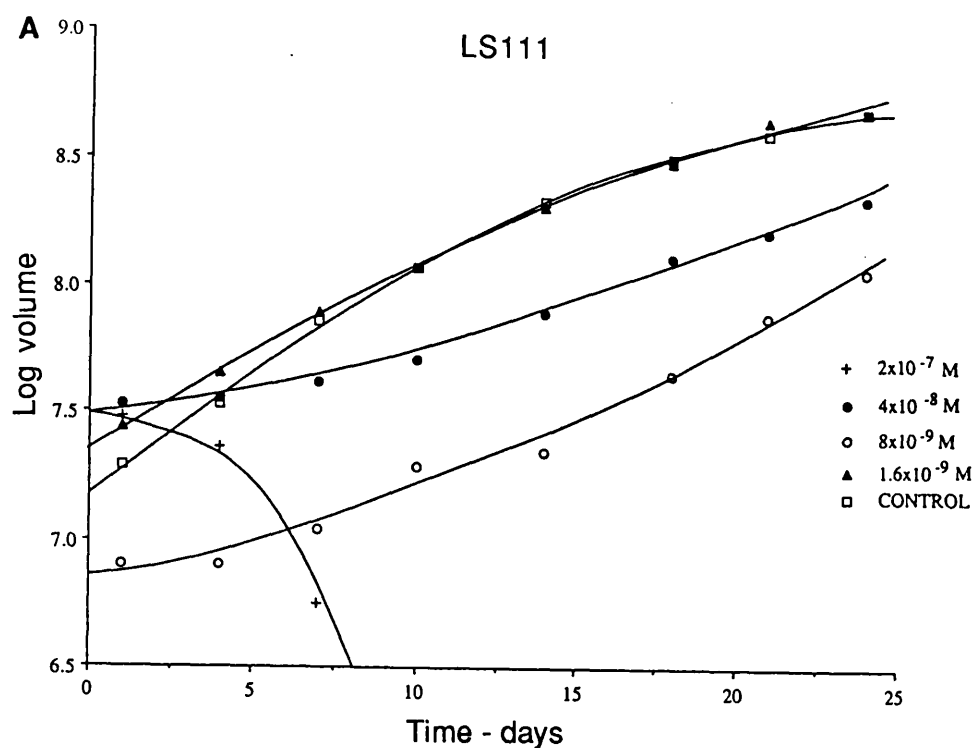


Figure 4.3

Aggregate volumes estimated at various times after exposure of cell-lines LS111 (A) and LS112FL (B) to doxorubicin for 24 hours. Values are the median ($n=24$).

DISCUSSION

It was a disappointment to find that only some of the newly established cell lines grew well in this system. The failure of some cell lines to proliferate may have been a reflection of their early age in tissue culture. Alternatively this may have been an indication of the requirement for the presence of mouse fibroblast (stromal) cells in the case of LCPH2 and LCPH3, as discussed in the preceding Chapter. A final possibility is that the aggregates required the presence of other aggregates in close proximity to stimulate continued growth, perhaps by means of unspecified growth factors (Minna et al., 1982).

Even in those cell lines where cytotoxicity testing was possible a number of problems became apparent. Firstly, in terms of setting up and running an experiment, the study of spheroid growth and growth delay was labour intensive. Secondly, the chemosensitivity tests were time consuming, lasting at least 3 weeks. Clearly this would not be ideal for the study of a large number of cell lines, different cytotoxic drugs and a number of resistance modifying agents.

In addition for any given cytotoxic drug concentration, the amount of growth delay appeared to be dependant on the starting size of the spheroid. Smaller spheroids appeared to be more sensitive to a given drug concentration. This point is emphasized in Figure 4.3A which shows similar growth delay in cell line LS111 at two different

drug concentrations ($8 \times 10^{-9}\text{M}$ and $4 \times 10^{-9}\text{M}$). This apparent discrepancy may perhaps be explained by the smaller median starting size of the spheroids at the lower drug concentration. Of course these comments relate to only a few observations but do serve to emphasize some of the apparent limitations of this technique. Certainly theoretically relatively small spheroids will have a greater proportion of cells in the outer region of the spheroid and cells in this region have been shown to be more sensitive to doxorubicin using a tritiated thymidine suicide technique in SCLC spheroids (Kwok & Twentyman, 1987). Despite many attempts (including the use of an eye-piece graticule) it proved very difficult to select constantly sized aggregates. This is simply a reflection of the spectrum in size of these natural SCLC aggregates at any one time. This is in contrast to the relatively uniform size of multi-cellular tumour spheroids generated artificially using agar-based flasks.

In addition, only SCLC aggregates from cell line LS111 had truly spherical geometry. Aggregates with intermediate or variant morphology grew in various branching shapes (Plate 4.3). Thus volume estimates became inaccurate. Moreover when the aggregates were killed by high drug concentrations, they became flat and although they appeared damaged, still generated a signal in the televisual computer system, so again leading to possible inaccuracies (Plate 4.4).

However, most of the above problems do not apply when this system is used to study laboratory generated multicellular tumour

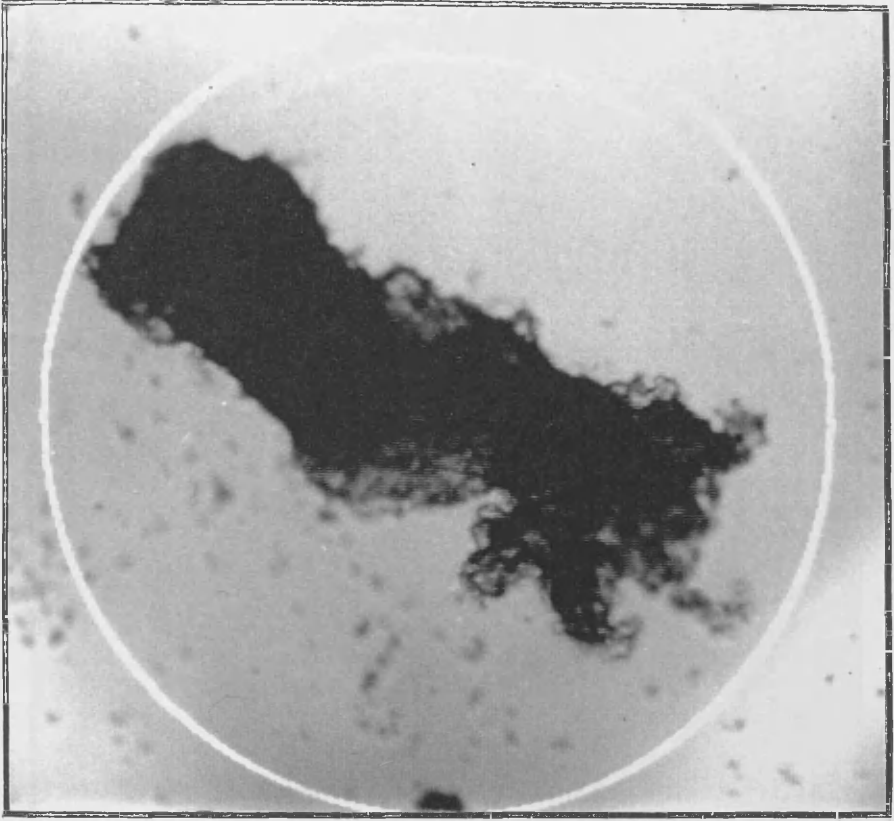


Plate 4.3

A tumour aggregate from cell line LS112FL. It can be seen that this representative aggregate does not possess spherical geometry.

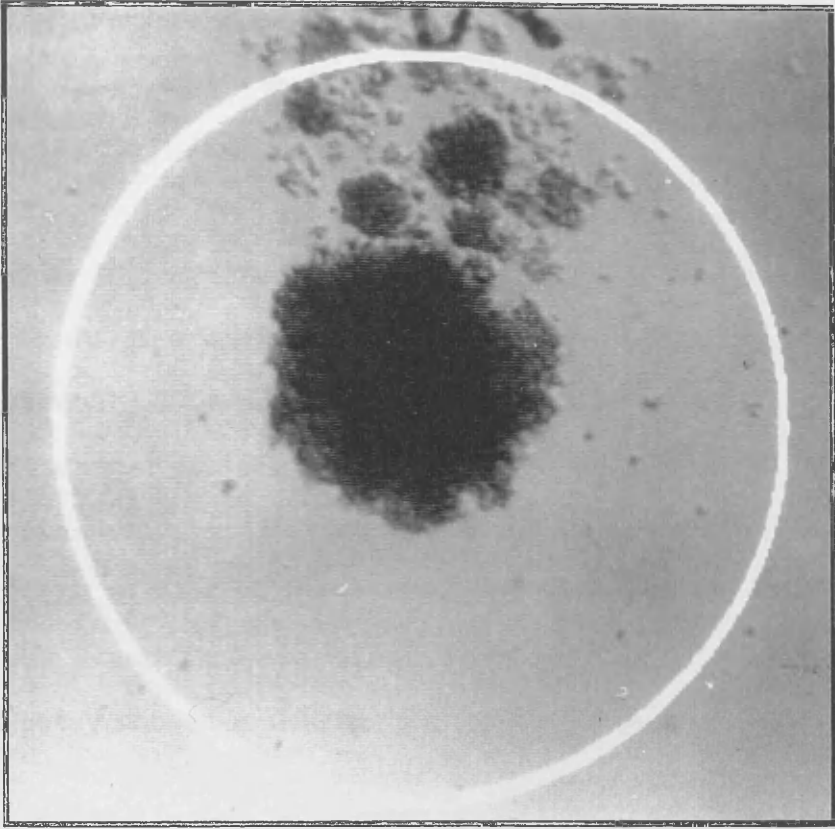


Plate 4.4

Estimation of the volume of an aggregate from cell line LS111 following exposure to doxorubicin ($2 \times 10^{-7}M$). Although the aggregate consists of dead cells, this structure still generates a signal.

spheroids. They possess spherical geometry, grow readily and rapidly in agar-coated plates and this allows accurate measurement of radiation and cytotoxic damage (Jones et al., 1982; Sutherland et al., 1979; West et al., 1980).

Thus this system did not appear suitable for the rapid and simple study of growth and chemosensitivity in a range of newly established SCLC lines. It was therefore necessary to re-consider some of the other systems used to measure chemosensitivity described in the Introduction to this chapter, in order to identify an assay that was suitable to measure the growth and chemosensitivity of the new SCLC cell lines. The adaptation of the metabolic (tetrazolium dye-based) assay chosen is described in the next Chapter.

CHAPTER 5

ADAPTATION OF A TETRAZOLIUM DYE-BASED ASSAY TO MEASURE CHEMOSENSITIVITY

INTRODUCTION

The difficulties of studying growth and chemosensitivity in cell lines which grow as floating aggregates were discussed in the previous Chapter. Recent studies from the National Cancer Institute of America reported the use of a tetrazolium dye-based assay in their drug screening programme (Alley et al., 1988; Carmichael et al., 1987). This assay seemed particularly attractive because it does not require disruption of aggregates into single cells, is rapid and easy to perform and can be semi-automated. It appeared to be reproducible and capable of detecting small changes in chemosensitivity. It therefore seemed suitable for adaptation to enable the study of growth and chemosensitivity in the newly established SCLC cell lines.

The assay is based on the principle that living metabolically active cells, but not dead cells, can reduce a water soluble yellow-coloured tetrazolium-dye (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide, MTT) to a water insoluble purple formazan product (MTT-F). The molecular structures of the tetrazolium dye

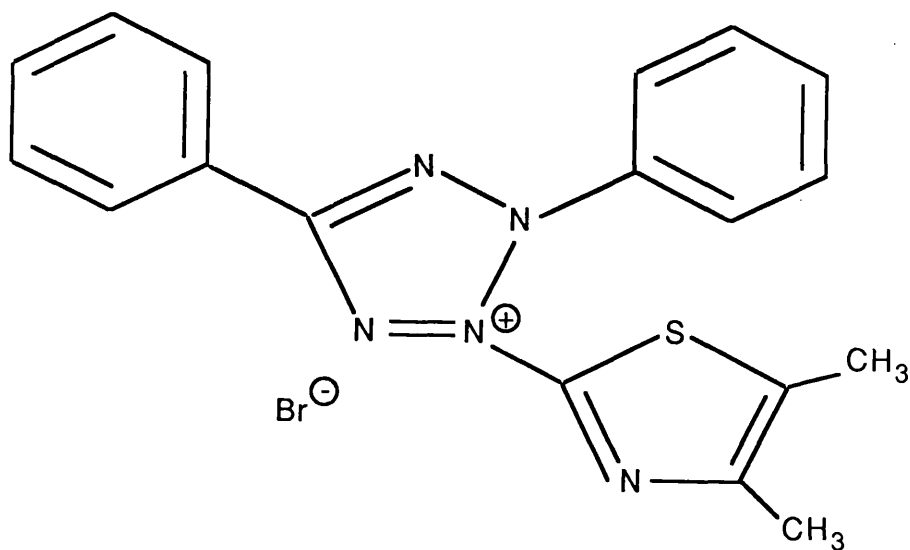
and the reduced form are shown in Figure 5.1. The tetrazolium ring is cleaved, probably by intracellular dehydrogenases (Hess & Pearce, 1963; Slater et al., 1963; Sowerby & Ottoway, 1962). This reduction produces the purple coloured MTT-formazan product.

Since the substrate (MTT) and the product (MTT-F) absorb at very different wavelengths no washing steps are required after removal of the aqueous medium before solubilisation of the MTT-formazan product. This is a clear advantage over those assays which use isotope incorporation as an end point, particularly when dealing with non-adherent cell lines.

History of Tetrazolium Dyes in Science

The tetrazolium salt MTT was first used almost 40 years ago to study the metabolism of normal and tumour tissues both in vitro and in vivo (Smith, 1951). Other workers subsequently used reduction of MTT by living cells to study metabolism in normal and tumour tissue (Black et al., 1953). Over the last two decades, many workers have used MTT reduction to determine the metabolic activity of viable cells (Schaeffer & Friend, 1976). Along with this use of MTT reduction to measure cell viability, went the assessment of toxic injury to cells by measuring decreases in MTT-F production. Black & Speer (1953) studied the activity of cytotoxic drugs in slices of excised tumour tissue using MTT reduction as an end-point. Herrmann et al. (1960) used MTT to detect virus induced

MTT



MTT - Formazan

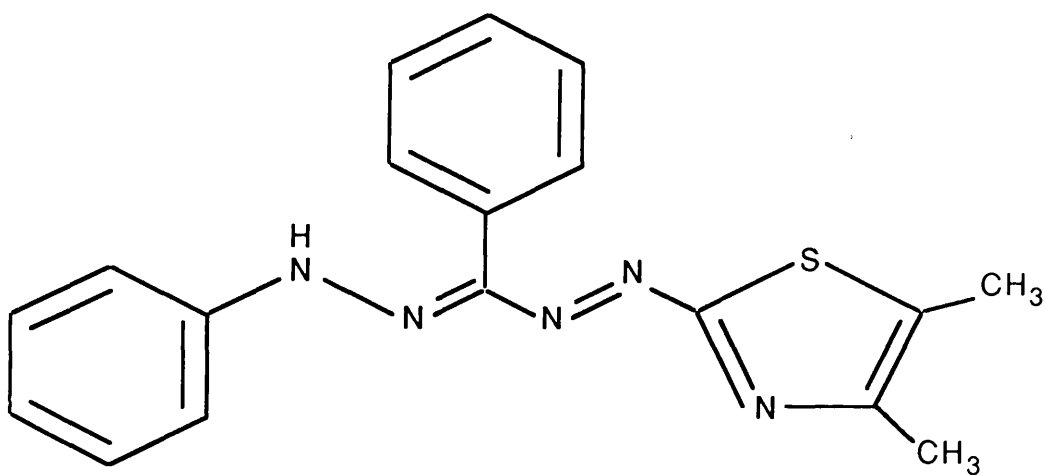


Figure 5.1

The structure of the tetrazolium dye, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and the reduced formazan product.

cytopathology in vitro and in 1967, Kondo & Ohkubo used MTT in a test to predict the effects of cytotoxic agents in vitro.

During the 1980s there was increasing interest in the use of MTT as a possible method of estimating cell injury in vitro. Mosmann (1983) and Green et al. (1984B) described how measuring the reduction in MTT-F production allows quantitation of cell damage following toxic injury. Alley has also described the use of MTT, initially to measure drug-induced cytotoxicity in soft agar colony forming assays (Alley et al., 1982) and in primary soft agar cultures of solid human tumours (Alley & Lieber, 1984). More recently he has been involved with workers at the National Cancer Institute to validate an automated microculture tetrazolium assay to study the growth and drug sensitivity of human tumour cell lines (Alley et al., 1988). However, this assay was designed for speed and simplicity in preference to accuracy.

The assay as originally described by Mosmann (1983) has been modified by a number of workers (Carmichael et al., 1987; Denizot & Lang, 1986) and recently Twentyman & Luscombe (1987) described a number of variables which affect the assay. In spite of these modifications a number of problems remain. In particular previous authors have failed to demonstrate a linear relationship between MTT-F production and cell number at high cell densities (Carmichael et al., 1987; Cole, 1986; Mosmann, 1983). In fact Ruben & Neubauer (1987) have recently highlighted this problem. They attribute their non-linear (hyperbolic) curves to reduced metabolic activity in wells which

have reached confluence. This point is particularly important since the highest cell numbers occur in the control, untreated wells and these are used to determine the parameters of sensitivity to the cytotoxic drug.

Other problems with the assay relate to the need to rationalise and define a time-course for the assay. This is particularly important with regard to the drug-exposure period and the subsequent recovery period. The factors which have to be considered include the mechanism of action of the drug and its stability in culture conditions. The recovery period is required to allow for damage which does not kill the cells but prevents cell division.

The modification of the tetrazolium assay is described in this Chapter. In order to study a number of variables adherent non-small cell lung cancer cell lines were used for the initial characterisation of the assay. The results from these cell lines were then extended to include well established non-adherent SCLC cell lines that could be disaggregated and counted. Finally, the modified assay was adapted for studies of the newly established small cell lung cancer cell lines.

METHODS

Cell Lines

Four non-small cell and two readily disaggregable small cell lung cancer cell lines were used initially to develop the assay. The non-small cell lines used were: L-DAN, a squamous lung cancer cell line established in our own laboratory; A549, a broncho-alveolar carcinoma cell line and SK-MES, a squamous lung carcinoma cell line both obtained from the American Type Tissue Collection and WIL, an adenocarcinoma cell line obtained from the Ludwig Institute for Cancer Research, Sutton, Surrey. These lines were maintained in a mixture of Ham's F10 and Dulbecco's Modified Eagle's Medium [DMEM] (50:50; Gibco Ltd., Paisley, Scotland) supplemented with glutamine (2mM) and foetal bovine serum (10% v/v) [F10/DMEM10%].

The two SCLC lines used were GLC4, kindly gifted by Dr E. de Vries, Department of Clinical Oncology, University of Groningen, The Netherlands and NCI-H187, (Carney et al., 1985). These lines were maintained in RPMI 1640 (Northumbria Biologicals Ltd., Cramlington, Northumberland, England) supplemented with glutamine (2mM) and foetal bovine serum (10% v/v) [RPMI 10%].

The culture conditions of the newly established SCLC cell lines have been described previously (Chapter 2).

Determination of Absorption Spectra

MTT and MTT-formazan (Sigma Chemical Company, Poole, Dorset) were dissolved in dimethylsulphoxide (DMSO) (BDH, Thornliebank, Glasgow). The absorbance was then scanned between wavelengths of 350nm and 700nm using a Gilford spectrophotometer. MTT-formazan produced by cells was obtained by incubation of cells (L-DAN) in the presence of MTT (5 mg/ml) for 4 hours, in the dark at 37°C in an atmosphere of 2% CO₂ in air. Any remaining MTT was removed and the formazan crystals were dissolved in DMSO and absorbance measured as described above.

Determination of Optimal MTT Concentration

Cells were plated out into 96 well plates in 200 µl of medium at a concentration of either 10⁴ cells per flat bottomed well (A549 and L-DAN; Linbro from Flow Laboratories, Irvine, Scotland) or 5 x 10⁴ cells per round bottomed well (GLC4 and NCI-H187; Nuncclon from Gibco, Paisley, Scotland). Cells were incubated for 24 hours at 37°C in an atmosphere of 2% CO₂ in air to allow attachment and/or growth. Subsequently MTT (at concentrations of between 0 and 5 mg/ml) was dissolved in PBS and 50 µl was added to each well, 8 wells per concentration. Plates were wrapped in aluminium foil and incubated for 4 hours at 37°C. Medium and any remaining MTT was carefully removed at the end of the incubation. Prior to removal of the medium

plates containing non-adherent cells were centrifuged at 200 g for 5 minutes. The formazan crystals produced were dissolved in 200 μ l of DMSO. In some experiments 25 μ l of Sorensen's glycine buffer (0.1M glycine + 0.1M NaCl equilibrated to a pH of 10.5 with NaOH 0.1N) was added at this stage. The absorbance values were recorded using an ELISA plate reader (Model 2550 EIA Plate Reader; Bio-rad Laboratories, Watford, England) at a wavelength of 570 nm.

Determination of Optimal Incubation Time

The non-small cell lung cancer cell lines A549 and SK-MES were plated out in 96 well microtitre plates and incubated for 24 hours as described above. MTT at the optimal concentration for each cell line (4 mg/ml for A549 and 4.25 mg/ml for SK-MES) was dissolved in PBS and 50 μ l was added to each well. Plates were incubated for 1, 2, 3 or 4 hours and then processed as described previously.

Relationship between MTT-Formazan Production and Cell Number

Cells (A549, L-DAN, GLC4 and NCI-H187) were diluted to a concentration of 2.5×10^5 cells per ml. Various volumes of this cell suspension (20 μ l-200 μ l) were plated out in duplicate 96 well microtitre plates (8 wells per volume), the final volume always being made up to 200 μ l with fresh medium. Plates were then incubated for 24 hours at 37°C in an atmosphere of 2% CO₂ in air. The duplicate

plates were then processed as follows. To one plate 50 μ l of MTT in PBS (concentration depending on cell line) was added and the plate incubated for 4 hours and processed as described above. The medium was removed from the other plate and 50 μ l trypsin/EDTA (0.25%/1mM) was added to each well. After incubation at 37°C for a few minutes the cells from 4 replicate wells were pooled and counted with an electronic counter (Coulter Electronics, Luton, England).

Growth Rates

Cells were plated out in 200 μ l of medium at a concentration of 10^3 per well (i.e. 5×10^3 /ml for A549 and L-DAN) or 5×10^3 per well (i.e. 2.5×10^4 /ml for GLC4 and NCI-H187). More than one cell line was plated out in each plate and separate plates were prepared for each time point. Plates were fed daily with fresh medium and every 2nd day one row (comprising eight wells) from a plate was trypsinized and counted as described above. MTT (50 μ l, concentration depending on cell line) was added to a second row and the plates processed as above.

Chemosensitivity Measurements

Microtitre Assay

Adherent cells (A549, L-DAN) were plated out at a concentration of 10^3 per well in 200 μ l of medium (i.e. 5×10^3

cells/ml) and allowed to attach and grow for 2 to 3 days. The medium was then removed from the wells and replaced with 200 μ l of medium (for the control wells) or 200 μ l of drug-containing medium. The cytotoxic drugs used were doxorubicin (Farmitalia Carlo Erba, St Albans, Herts.), and vincristine (Sigma Co. Ltd., Poole, Dorset). Drug was always freshly made up and diluted in culture medium. Cells were incubated with drug for 24 hours after which the drug-containing medium was replaced by drug-free medium. Plates were then fed daily with 200 μ l of fresh medium for 2 days. On the fifth day following drug addition the cells were fed with 200 μ l of fresh medium containing HEPES buffer (10mM, pH 7.4) to maintain a constant pH during MTT incubation. MTT (50 μ l of a pre-determined concentration) was added to the wells and the plates were incubated and processed as described previously. In all experiments in which chemosensitivity was measured by microtitre assay, drug sensitivity was defined as that concentration of drug required to reduce absorbance to 50% of control absorbance (ID_{50}).

For the non-adherent cell lines (GLC4 and NCI-H187) exponentially growing cells were plated out at a concentration of 5×10^3 cells per well in 100 μ l of culture medium. Drug was added immediately in a volume of 100 μ l at twice the desired final concentration. Cells were exposed to drug for 24 hours and then fed and processed as described above. Plates containing the non-adherent SCLC aggregates had to be centrifuged at 200g for 5 minutes every day prior to feeding.

Clonogenic Assay

Cells (A549 and L-DAN) were plated out at a concentration of 7.8×10^4 cells per 25 cm² flask (Nunc; Gibco, Paisley, U.K.). This gave the same cell density/cm² as was used for the microtitre assay. The cells were allowed to attach and grow for 2 to 3 days. Flasks were then fed with 5 mls of fresh medium or medium containing drug and incubated for 24 hours. The medium was then removed and the cells washed with 5 mls of trypsin/EDTA (0.25 %/1mM) and incubated for a few minutes at 37°C. Cells were then resuspended in 10 mls of fresh medium and the cells in the control, untreated flasks counted. They were diluted to 100 cells per ml and 5 mls was plated out into each of 4 Petri Dishes (6 cm, Nunc; Gibco, Paisley, Scotland). Cells from the drug-treated flasks were diluted in the same way as the control flask and plated out as above. The dishes were incubated at 37°C in an atmosphere of 2% CO₂ in air for 10 days. Medium was then removed, the Petri dishes were washed with PBS, fixed in methanol and stained with crystal violet. Colonies of greater than 50 cells were counted.

Effect of Time after Drug Exposure on the Apparent Chemosensitivity of the Cells

Cells (A549) were plated out, and exposed to various concentrations of doxorubicin for 24 hours, as described above. Thereafter, plates were fed daily for 24-96 hours. Residual cell

viability was estimated by MTT reduction as previously described, at 24,48,72 and 96 hours after drug exposure.

**Doxorubicin Penetration into and MTT-Formazan Production by
Aggregates from Newly Established SCLC Cell Lines**

Aggregates from each of the newly established SCLC cell lines were incubated with either doxorubicin (10^{-6} M) or MTT (5mg/ml) for 4 hours. The aggregates were then washed once with cold PBS and then mounted in OCT (BDH Ltd., Thornliebank, Glasgow) on cryotome blocks resting in dry ice. Frozen sections (5 micron) were prepared with a cryostat. These sections were observed unmounted to assess the viable cell population. Subsequently sections were mounted in "U V Inert" (BDH Ltd., Thornliebank, Glasgow). Those incubated in doxorubicin were mounted in glycerol and viewed by fluorescent microscopy. Those aggregates incubated in MTT were examined by light microscopy.

RESULTS

Spectral Analysis

The absorption spectra of MTT and MTT-formazan are shown in Figure 5.2. Also shown is the absorption spectrum for MTT-formazan produced by cells incubated in the presence of MTT. For MTT there is a single absorption maximum at a wavelength of 410 nm. Pure MTT-formazan obtained from the manufacturing chemist exhibits two absorption maxima at wavelengths of about 510 nm and 570 nm. In contrast, MTT-formazan produced by cells (4×10^3 /well, L-DAN) incubated with MTT (5 mg/ml) demonstrates a single absorption maximum at a wavelength of about 570nm.

Determination of Optimal MTT Concentration

Figure 5.3A shows the amount of MTT-formazan produced when the cell line L-DAN is incubated with various concentrations of MTT. MTT-formazan production increases with increasing MTT concentration up to 4.5 mg/ml. For A549 a plateau of MTT-formazan production is reached with an MTT concentration of 4.2 mg/ml (data not shown). For the two SCLC cell lines MTT-formazan production was maximal at much lower MTT concentrations (1.8 mg/ml for GLC4, Fig 5.3B, and 1.2 mg/ml for NCI-H187, Fig 5.3C). In no case were MTT concentrations of up to 5 mg/ml toxic to the cells.

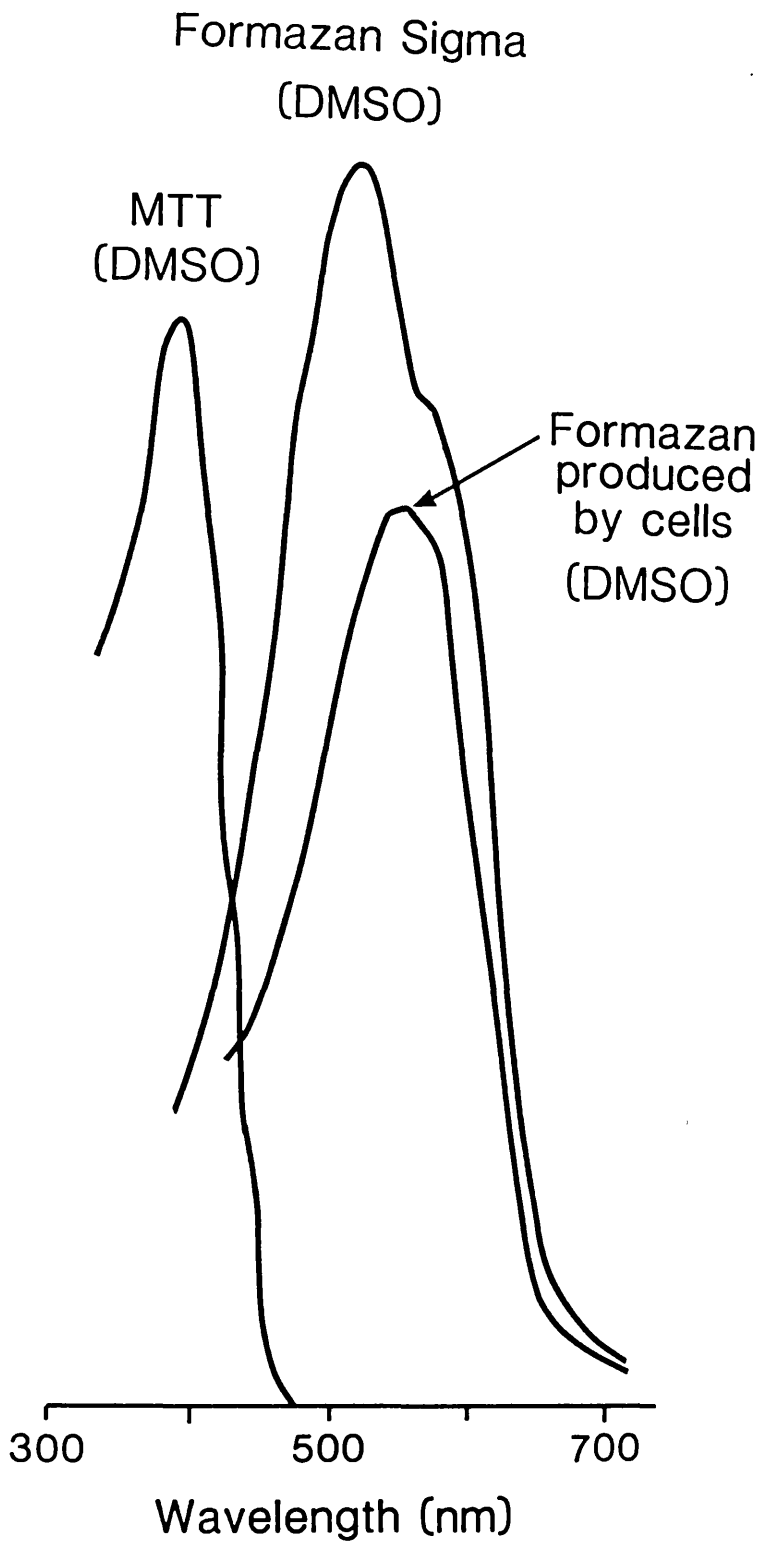


Figure 5.2

Absorption spectra of MTT and commercially prepared MTT-formazan dissolved in DMSO. Also shown is the spectrum of MTT-formazan produced by incubation of L-DAN cells (4,000 per well) with MTT (5mg/ml) for 4 hours.

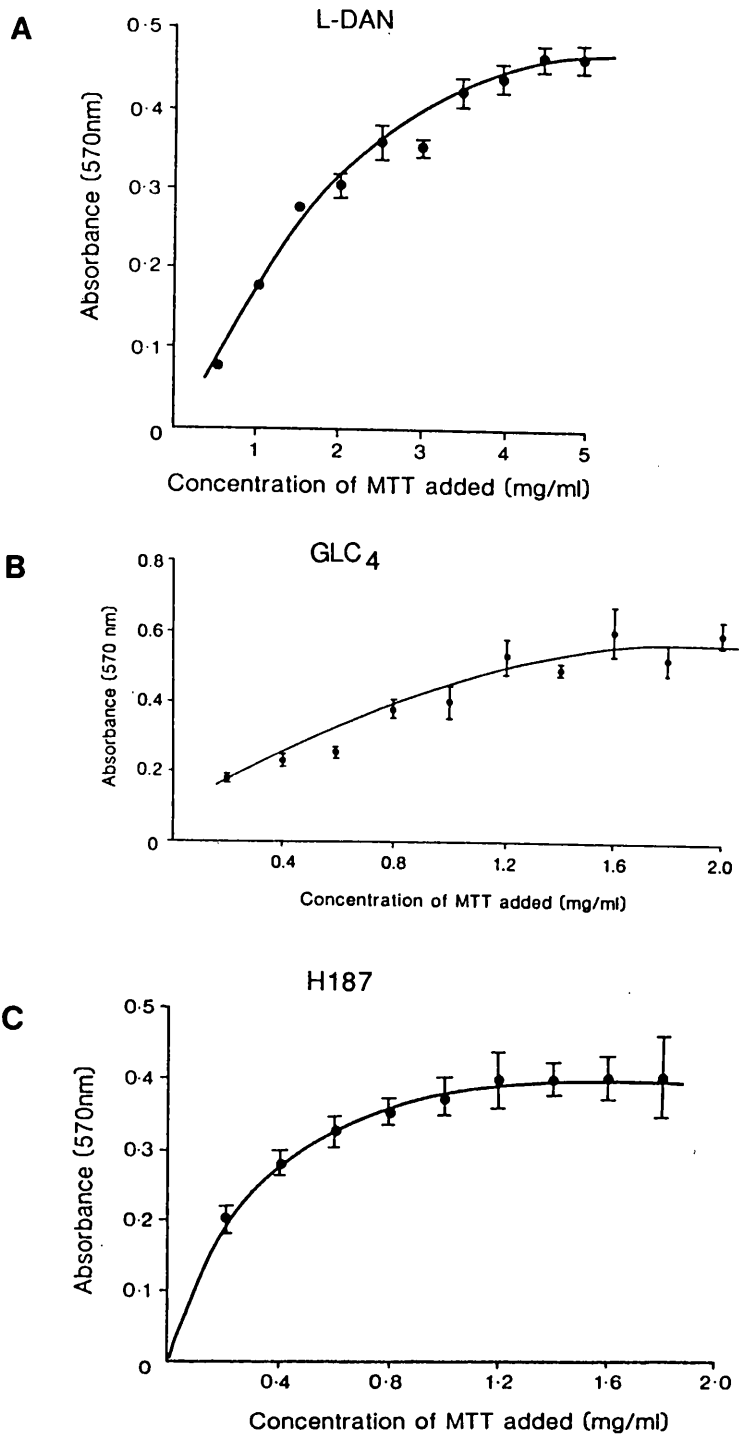


Figure 5.3

Effect of MTT concentration on MTT-formazan production. Cells, L-DAN (A), GLC₄ (B) or NCI-H 187 (C) were plated out at a density of 5×10^4 per well and allowed to grow for 24 hours. MTT (50ul of a solution of 0 to 5 mg/ml) was added (8 wells per concentration), and the plate was incubated for 4 hours. The medium was removed, and the MTT-formazan crystals were dissolved in DMSO. Values are the mean \pm Standard Error of the Mean (SEM) of 8 observations.

Determination of Optimal Incubation Time

Figure 5.4 shows that for both cell lines studied [A549 (A) and SK-MES (B)] the amount of MTT-formazan produced increases during the first 3 hours of incubation. MTT-F production then appears to plateau.

Relationship between MTT-Formazan Production and Cell Number

The relationship between MTT-formazan production and cell number for the cell lines L-DAN (A) and GLC4 (B) is shown in Figure 5.5. Although there is a clear linear relationship up to a cell number of about 2×10^4 cells per well, in neither case does the regression line extrapolate through the origin.

Effect of Cell Number on the Absorption Spectrum of MTT-Formazan

Figure 5.6 shows the absorption spectrum of MTT-formazan produced by cells following incubation of A549 cells with MTT (5 mg/ml) for 4 hours at low (4×10^3 cells/well) and high (10^4 cells/well) cell densities. For low cell numbers the spectrum shows a single absorption maximum at a wavelength of about 570 nm. In contrast, at higher cell numbers the peak is much broader with the absorption maximum at a wavelength of about 510 nm and a shoulder in this peak at about 580 nm.

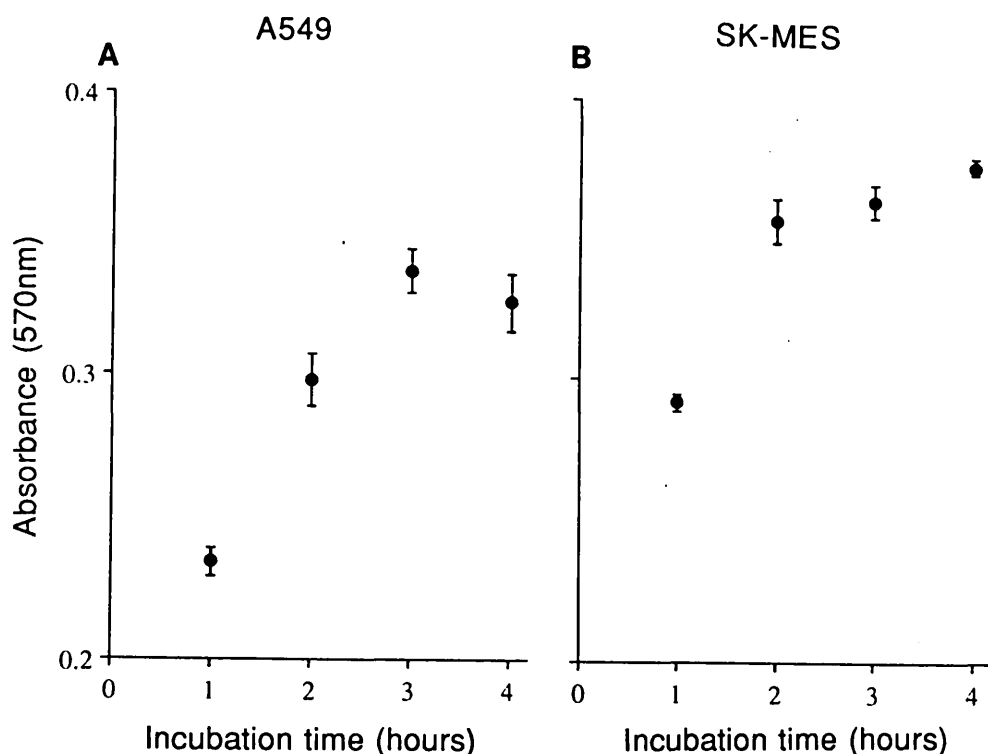


Figure 5.4

Determination of optimal incubation time for MTT. Cells, either A549 (A) or SK-MES (B) were plated out at a density of 10^4 per well and allowed to grow for 24 hours. MTT (50ul) at the optimal concentration for each cell-line (4 mg/ml for A549 and 4.25 mg/ml for SK-MES) was added to each well and plates were incubated for 1-4 hours. The medium was removed, and the MTT-formazan crystals were dissolved in DMSO. Values are the mean \pm SEM of 8 observations.

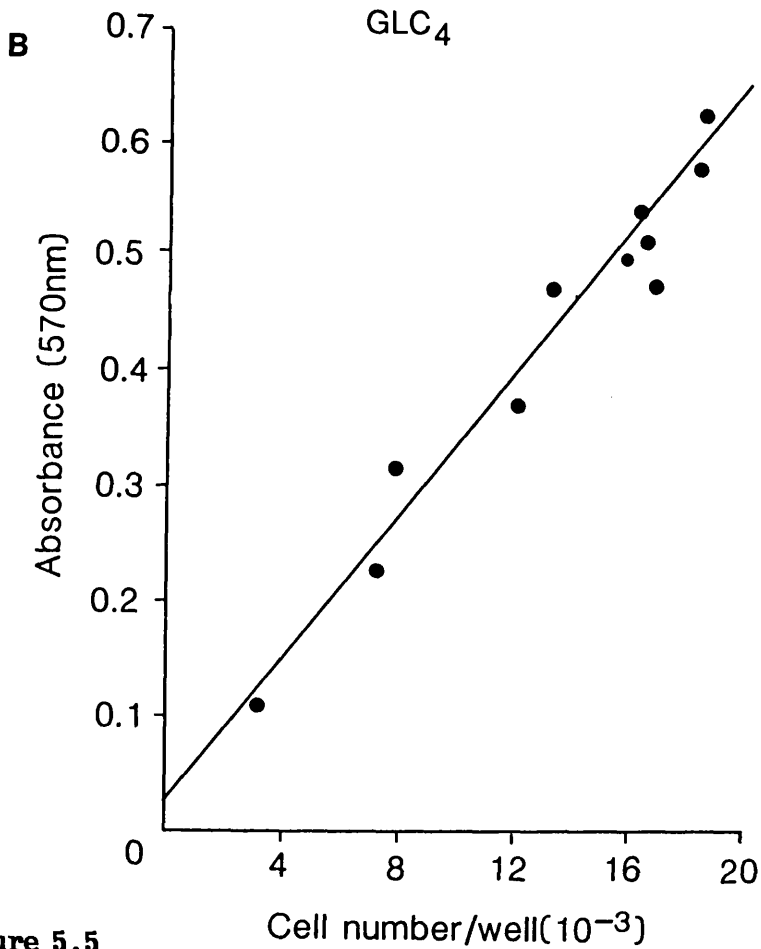
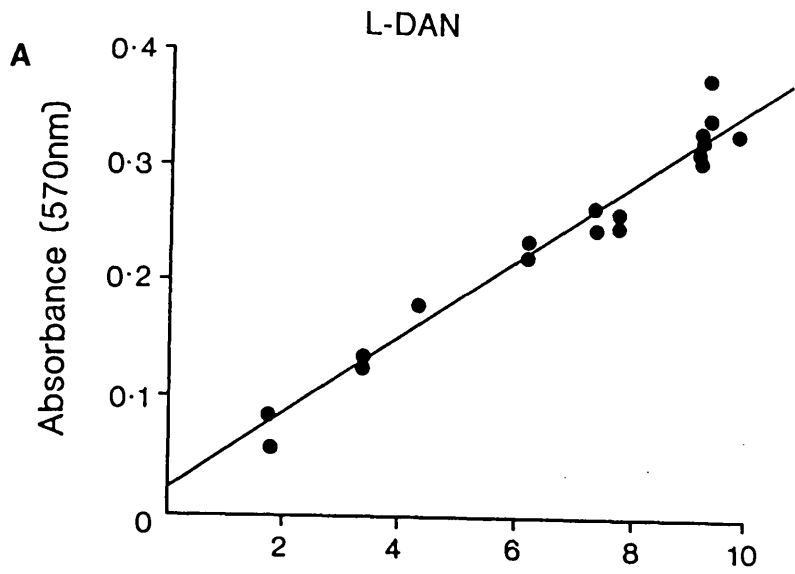


Figure 5.5

Relationship between MTT-formazan production and cell number for the cell lines L-DAN (A) and GLC₄ (B). Cells were plated out at a range of concentrations and incubated for 24 hours. Cell number was then estimated either by counting following trypsin treatment if required or by incubation with MTT (L-DAN, 5mg/ml; GLC₄, 2 mg/ml) for 4 hours.

FORMAZAN PRODUCED BY CELLS

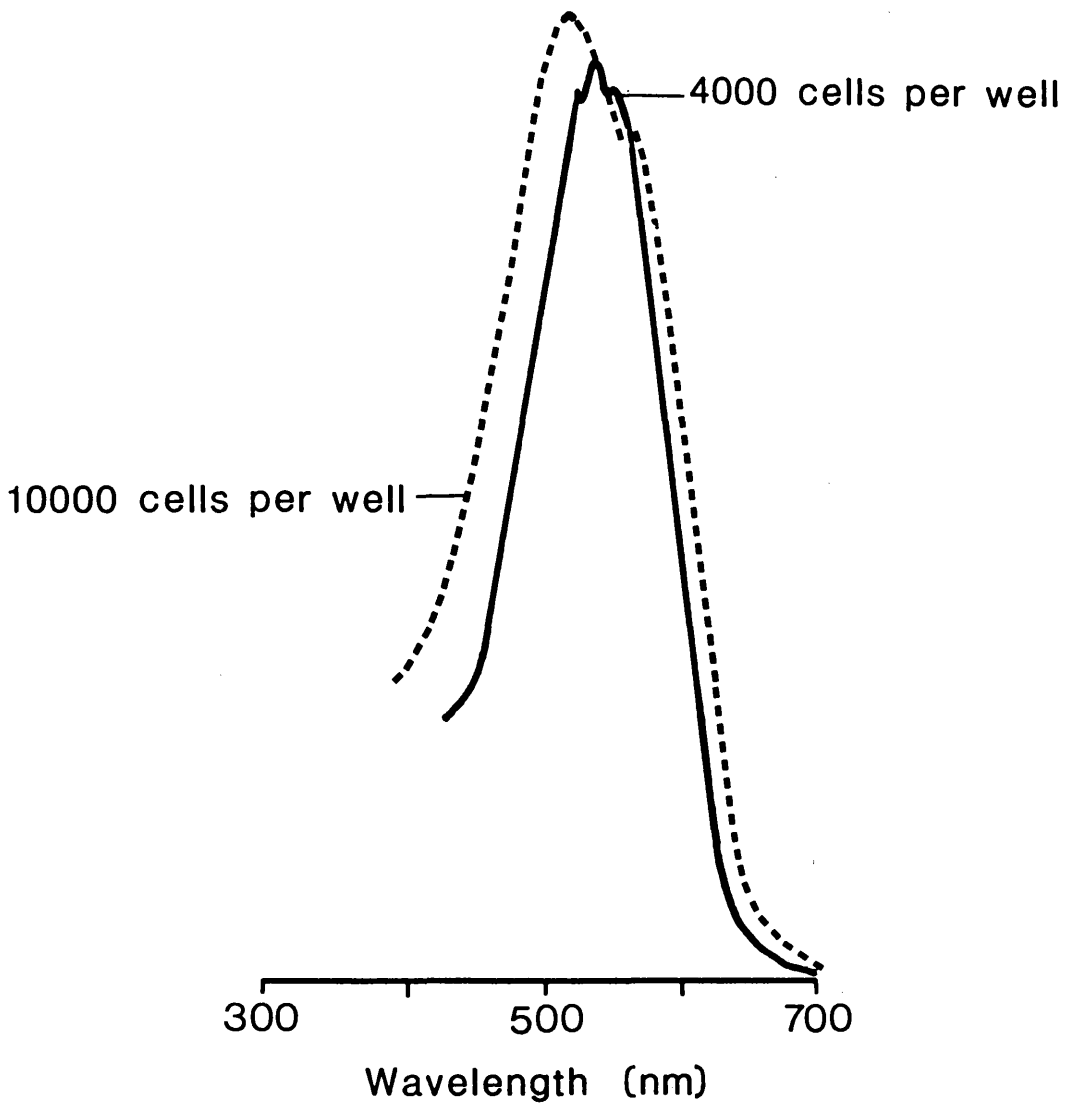


Figure 5.6

Absorption spectrum of MTT-formazan produced by incubation of A549 cells with MTT (5 mg/ml) for 4 hours at low (4×10^3 cells per well) and high (10^4 cells per well) cell densities. MTT-formazan crystals were dissolved in DMSO.

Effect of pH on MTT-Formazan Absorbance

Figure 5.7 shows the spectral analysis of solutions of MTT-formazan in DMSO (200 μ l) in the presence of glycine buffer (25 μ l) equilibrated at a range of pH values. Addition of buffer resulted in increased absorbance values for the MTT-formazan in all cases. The increase was small at physiological pH (7.4). At higher pH values the absorbance increased with increasing pH, and reached a maximum at a pH of 10.5. There was no further increase in absorbance at pH values greater than 10.5

Effect of Buffer Addition on the Relationship between MTT-Formazan Production and Cell Number

Figure 5.8 shows the relationship between MTT-formazan production and cell number for the cell line A549 both before (open circles) and after (closed circles) addition of buffer to the wells (25 μ l, 0.1M, pH 10.5). The absorbance values are increased for all cell numbers but the increase is most marked at high cell numbers. Furthermore, following addition of buffer the regression line extrapolates through the origin.

HEPES buffer (10 mM, pH 7.4) was added during the period of incubation with MTT to control the pH. This did not alter the gradient of the line but did reduce the scatter of points about the line (Figure 5.9).

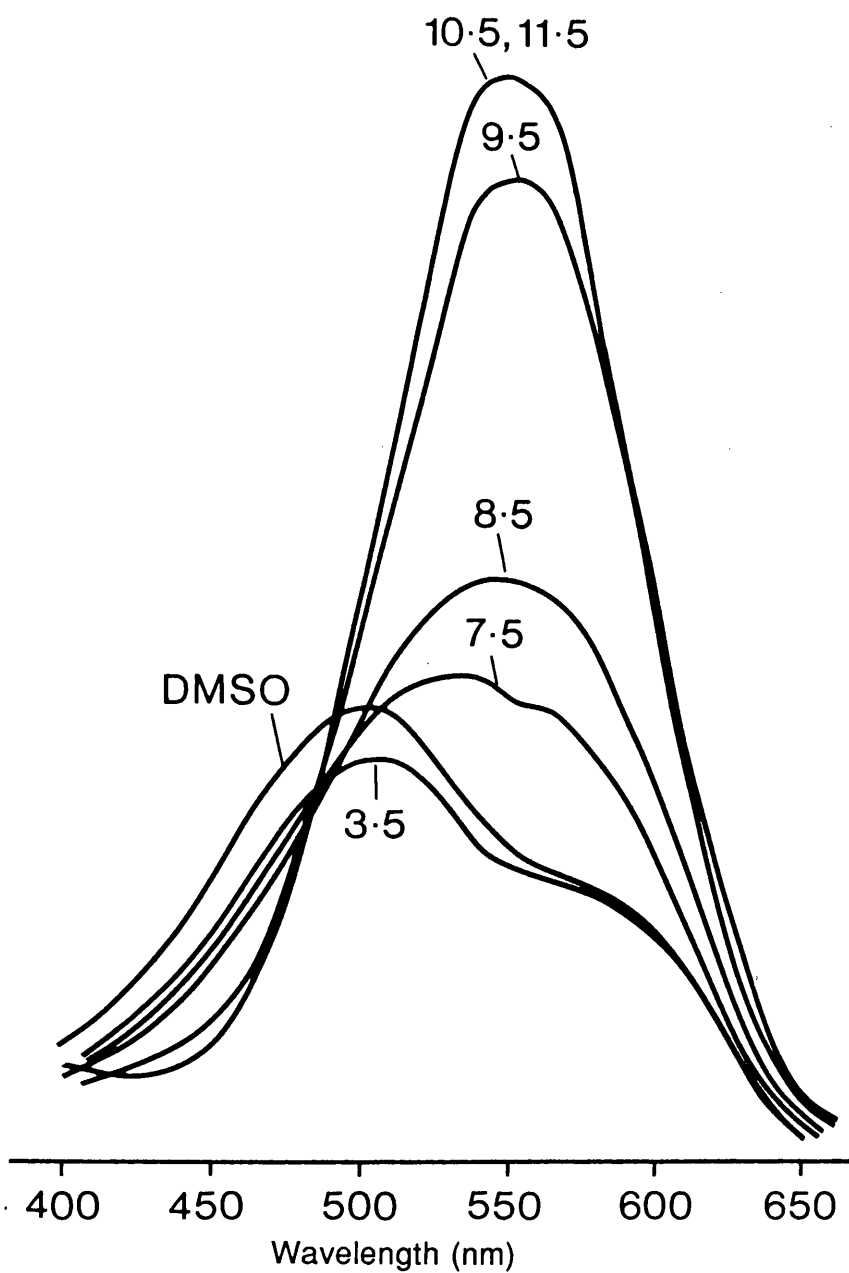


Figure 5.7

Absorption spectrum of a solution of commercially prepared MTT-formazan in DMSO (200ul) or in DMSO (200ul) plus glycine buffer (25ul) equilibrated at a range of pH values. For pH 7.5, a phosphate buffer was used.

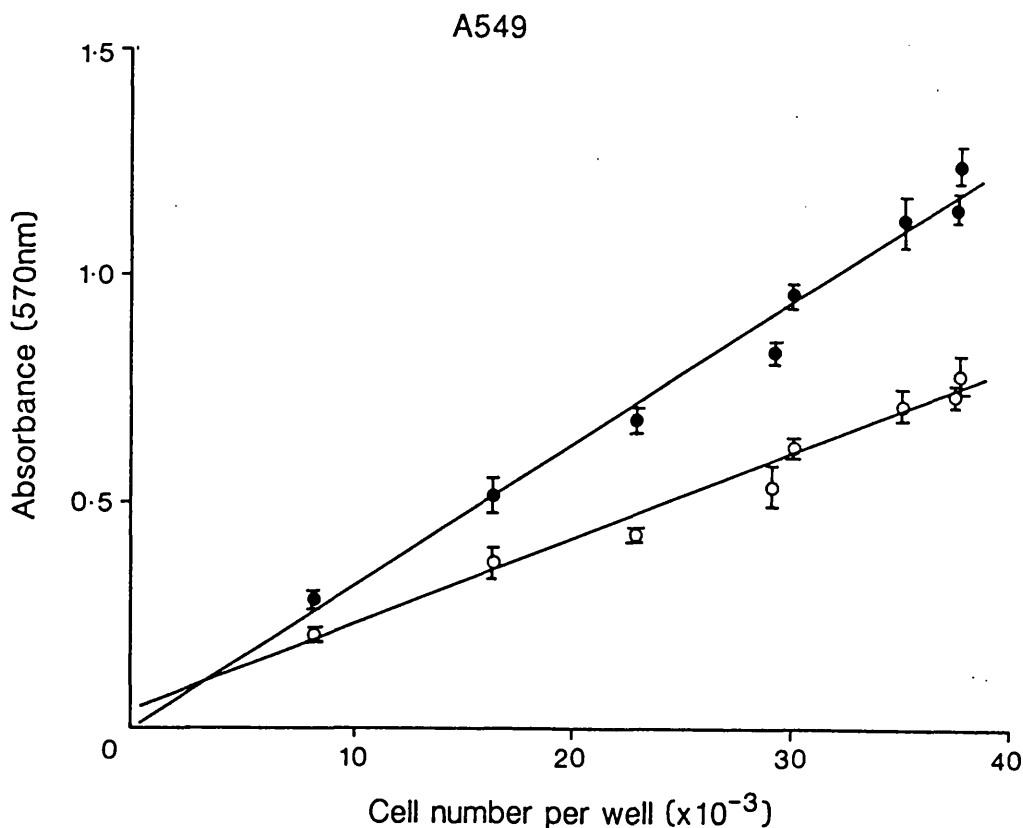


Figure 5.8

Effect of buffer addition on the relationship between MTT-formazan production and cell number. Cells (A549) were plated out at a range of concentrations and allowed to adhere for 24 hours. Cells were then either trypsinized and counted or incubated with MTT (5mg/ml) for 4 hours. Medium was removed, and MTT-formazan crystals were dissolved in DMSO, and the absorbance was recorded at 570 nm (O). Buffer (25ul, 0.1 M glycine, pH 10.5) was added to each well, and the absorbance was again recorded at 570 nm (●).

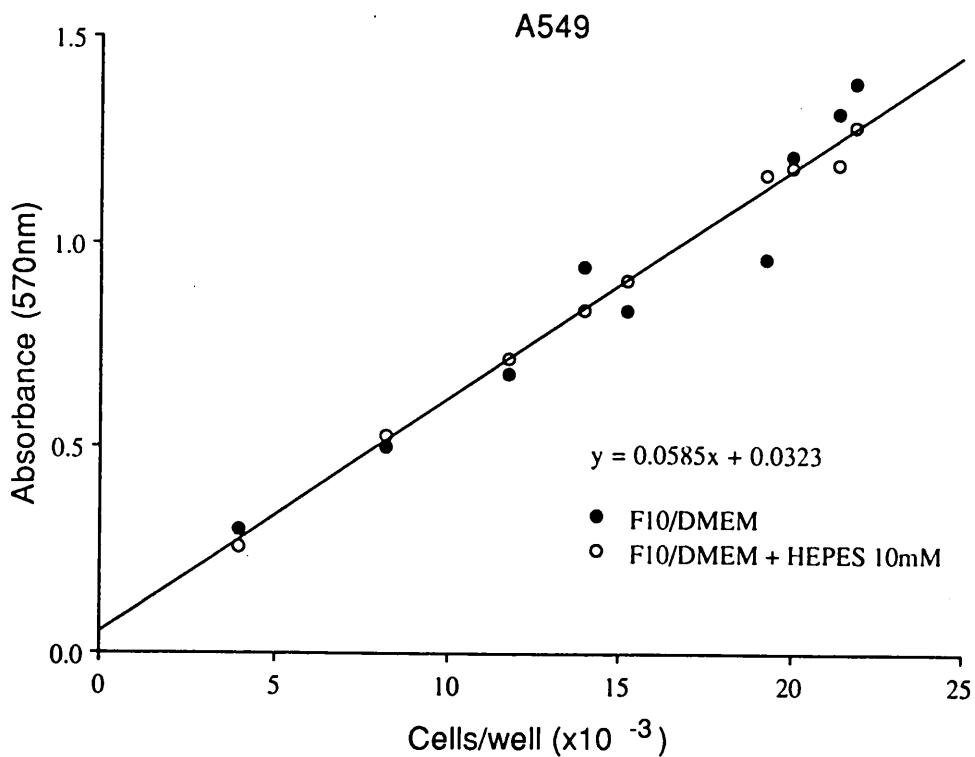


Figure 5.9

Effect of addition of HEPES buffer during MTT incubation. Cells (A549) were plated out at a range of concentrations and allowed to adhere for 24 hours. Cells were then either trypsinized and counted or incubated with MTT (5 mg/ml) for 4 hours with (O) or without (●) HEPES buffer (10 mM, pH 7.4).

Effect of Culture Medium on MTT-Formazan Production

Table 5.1 shows the effect of different volumes of culture medium (F10/DMEM 10%) on the absorbance of a standard solution of MTT-formazan. Addition of culture medium results in an increase in the absorbance of the MTT-formazan. Interestingly the increase in absorbance values is greater for small additions than for large additions of medium.

This table also shows the absorbance values obtained after addition of buffer (25 ul per well, 0.1M, pH 10.5) to the same plate. Although there is still a slight increase in absorbance in the presence of 5 ul of culture medium, addition of up to 50 ul of medium results in only a very small reduction in MTT-formazan absorbance. It should be noted that the addition of buffer alone to MTT-formazan resulted in a marked increase in the absorbance value.

Growth Studies

Growth curves for the non-small cell (L-DAN, A) and small cell (GLC4, B) lung cancer cell lines are shown in Figure 5.10. Doubling times of 24 hours for both cell lines compare well with those obtained by cell counts.

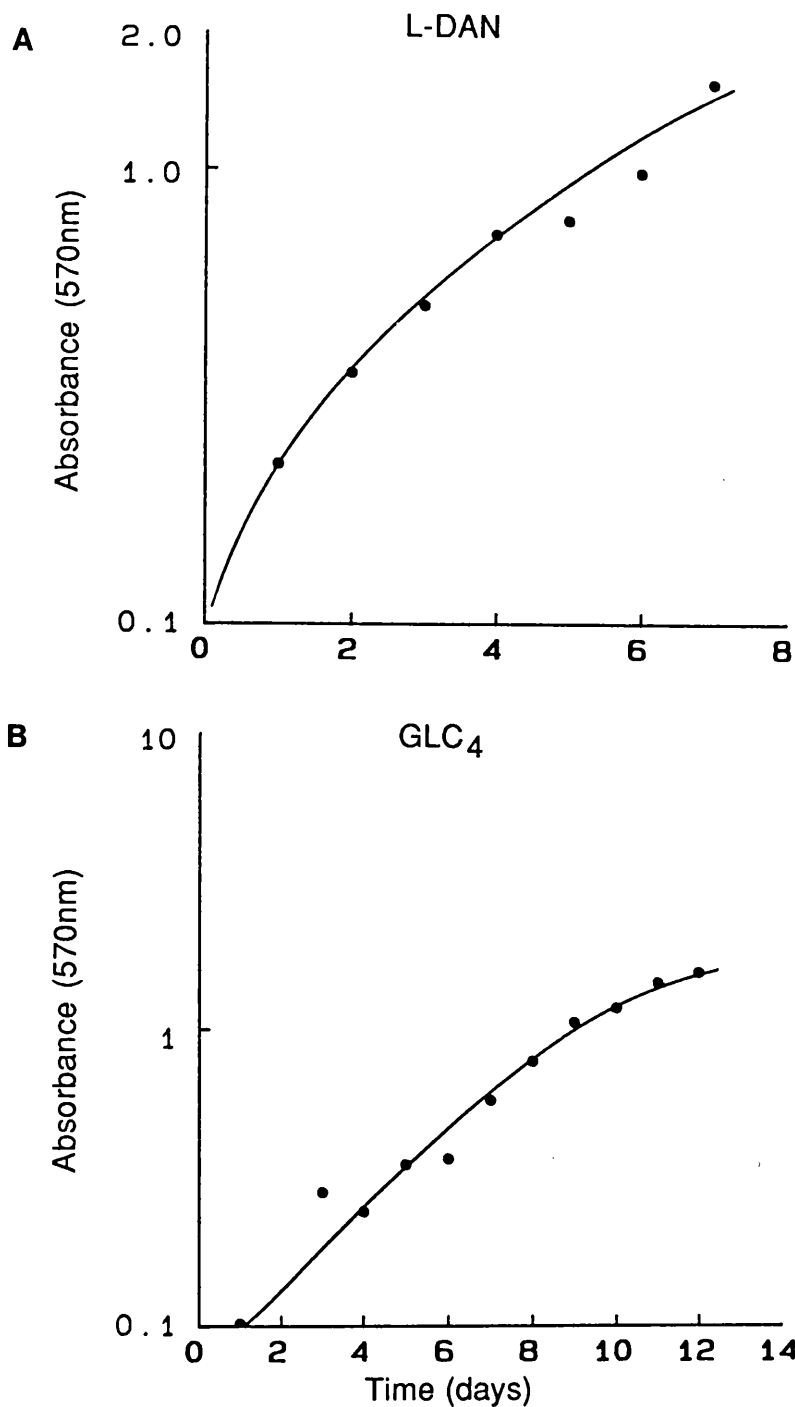


Figure 5.10

MTT-formazan production per well determined at various times after plating out L-DAN cells (A) and GLC₄ cells (B) at a density of 10^3 cells/well in 96-well plates. Cells were fed daily and at specified times MTT (50ul; 5mg/ml for L-DAN, 2mg/ml for GLC₄) was added to one plate. MTT-formazan crystals were dissolved in DMSO, and after addition of buffer (25ul, 0.1M glycine, pH 10.5) absorbance was noted at 570 nm. Results are the mean of 8 wells.

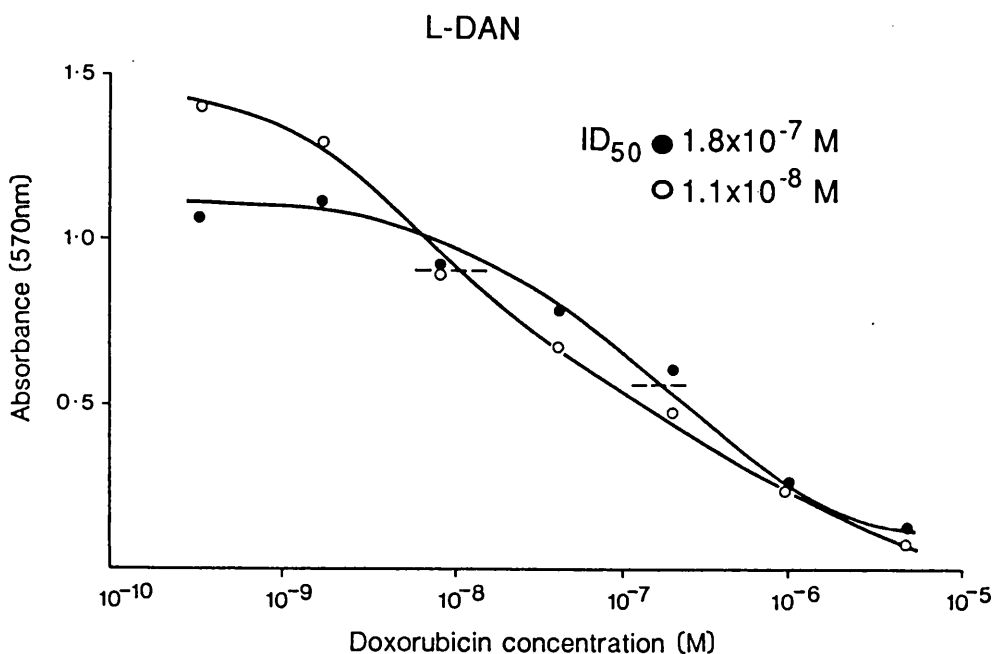


Figure 5.11

MTT-formazan production per well determined 3 days after exposure of cells (L-DAN) to various concentrations of doxorubicin for 24 hours. MTT-formazan crystals were dissolved in DMSO, and the absorbance noted before (●) and after (○) addition of buffer (25ul, 0.1M glycine, pH 10.5). ID_{50} indicated (----), represents that concentration of doxorubicin required to reduce absorbance values by 50%. Values are the mean of 8 wells.

Recovery time (days)	ID50 (nM)
1	140 \pm 28
2	120 \pm 22
3	38 \pm 7
4	38 \pm 6

Table 5.2

Sensitivity of A549 to doxorubicin (ID50, nM) determined by the microtitration assay and estimated at various times after drug removal. Results are the mean +/- SEM of 6 estimations.

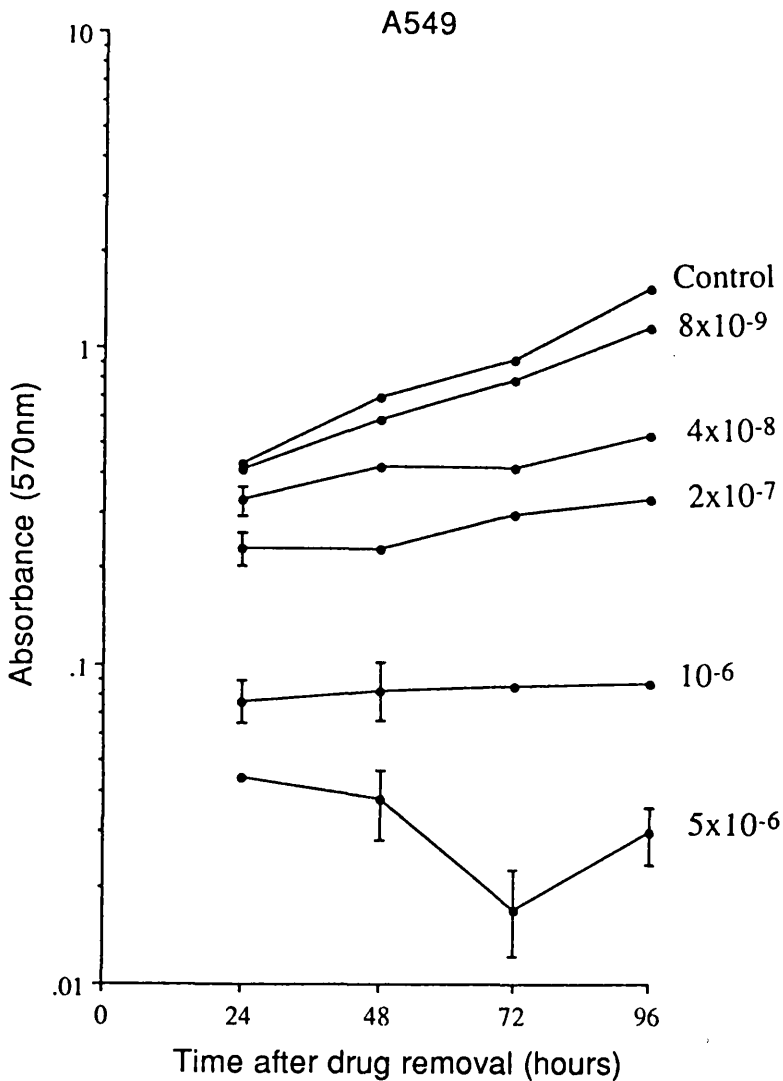


Figure 5.12

Effect of time after drug exposure on the apparent chemosensitivity of tumour cells. Cells (A549) were plated out at a density of 10^3 per well and were allowed to attach and grow for 2 days. Cells were then exposed to varying concentrations of doxorubicin (8×10^{-9} M - 5×10^{-6} M) for 24 hours. Drug containing medium was then removed and cells were fed daily for 1-4 days. Residual cell viability was estimated by MTT reduction at specified times after drug exposure. Values are the mean of 8 wells.

Effect of Buffer Addition on the Apparent Chemosensitivity to Doxorubicin

Figure 5.11 shows MTT-formazan production per well 4 days after exposure of L-DAN cells to various concentrations of doxorubicin for 24 hours. When the MTT-formazan crystals were dissolved in DMSO and absorbance values were measured at 570 nm, the ID_{50} was $1.8 \times 10^{-7} M$. However, following addition of buffer (25 μ l per well, 0.1M, pH 10.5) the absorbance at 570 nm was greatly increased in the control (untreated) wells and in those wells exposed to a low drug concentration; in this case the ID_{50} was $1.1 \times 10^{-8} M$ indicating an apparent increase in the sensitivity of the cells to doxorubicin.

Effect of Recovery Time on Chemosensitivity

Table 5.2 shows the sensitivity to doxorubicin determined by microtitration assay for the cell line A549. Cells were exposed to drug for 24 hours and MTT was added at various times after drug exposure. The apparent sensitivity of the cell line increases with increasing recovery time over the first 3 days after drug removal. After 3 days there is no further increase in sensitivity. Figure 5.12 shows the absorbance per well determined at various times after removal of the cytotoxic drug (doxorubicin) for A549 cells. At low drug concentrations ($6.4 \times 10^{-11} - 8 \times 10^{-9} M$) the cell number and

growth rate is the same as for the control untreated cells. There is a significant reduction in cell numbers 24 hours after drug removal at all higher drug concentrations. Although some growth is observed at 4×10^{-8} M and 2×10^{-7} M of doxorubicin, the growth rate is much slower than for the control cells. At all higher drug concentrations no growth or cell death is observed.

Determination of Chemosensitivity by the Microtitre Assay and Correlation with a Clonogenic Assay

Figure 5.13 shows representative chemosensitivity profiles to doxorubicin for the cell lines L-DAN (A) and GLC4 (B). Table 5.3 shows the chemosensitivities of the two SCLC cell lines to doxorubicin. Also shown in Table 5.3 are the sensitivities of the two non-small cell lung cancer cell lines to both doxorubicin and vincristine determined both by the microtitration assay and by the standard clonogenic assay described above. The standard errors for the two assays are similar and there is close agreement between the two assays for both cell lines and for both drugs. The overall error of the estimates in the microtitre assay is small (Co-efficient of Variation = 19%, Table 5.4) and is similar for both the adherent and non-adherent cell lines.

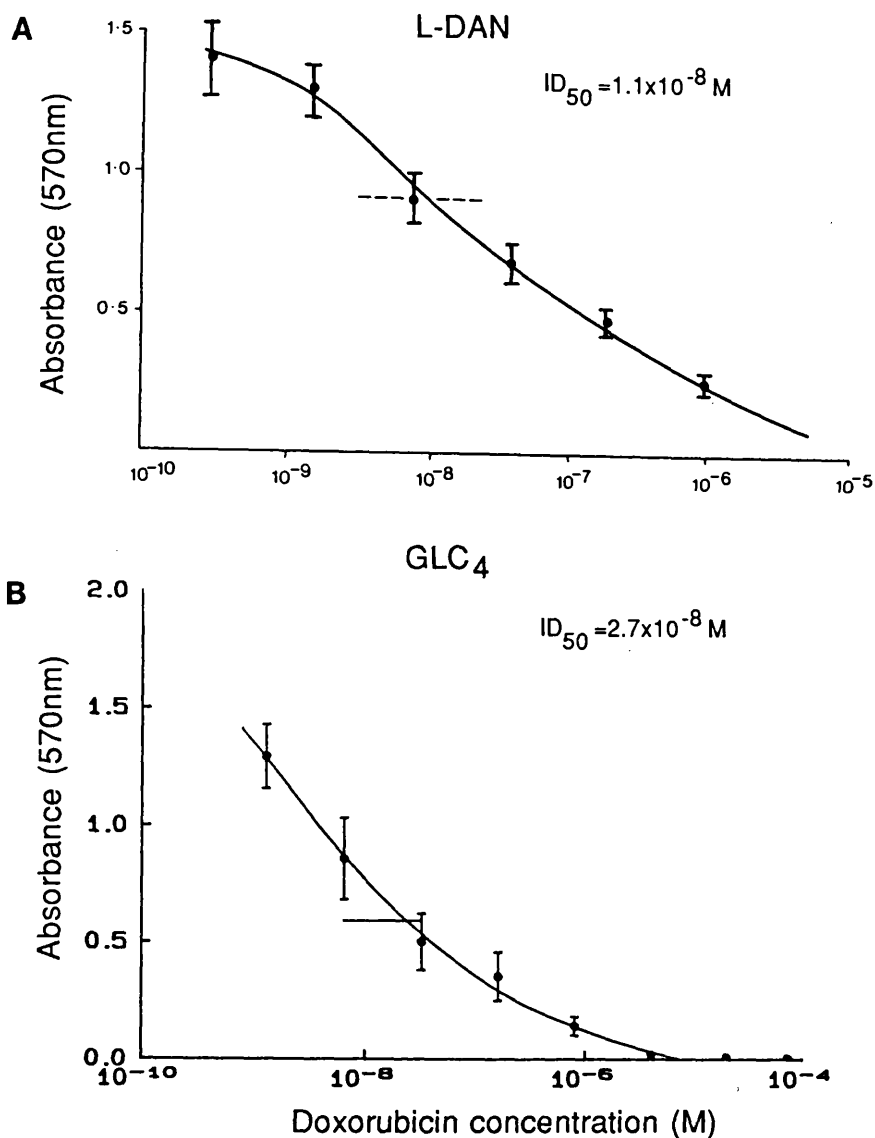


Figure 5.13

Determination of chemosensitivity to doxorubicin for L-DAN (A) and GLC₄ (B). For L-DAN, cells were plated out (10^5 per well) and allowed to attach and grow for 2-3 days, and then exposed to various concentrations of doxorubicin for 24 hours. For GLC₄, cells were plated out (5×10^5 per well) and exposed to various concentrations of doxorubicin for 24 hours. Subsequently drug containing medium was removed, and cells were fed daily. On the fifth day following drug addition remaining viable cell number was estimated by MTT reduction. Values are the mean \pm SEM of 8 observations.

Drug	Cell Line	ID50 (nM)	
		Microtitration	Clonogenic
Doxorubicin	NCI-H187	36* \pm 2 (6)**	-
	GLC4	12 \pm 3 (6)	-
	L-DAN	23 \pm 4 (7)	24 \pm 7 (6)
	A549	23 \pm 4 (7)	38 \pm 7 (6)
	WIL	57 \pm 6 (7)	34 \pm 8 (4)
	SK-MES	33 \pm 2 (10)	21 \pm 7 (4)
Vincristine	L-DAN	12 \pm 1 (6)	12 \pm 2 (6)
	A549	44 \pm 10 (5)	22 \pm 4 (5)
	WIL	41 \pm 4 (7)	20 \pm 8 (4)

* Mean \pm SEM

** Numbers in parentheses, number of estimates

Table 5.3

Chemosensitivity (ID50, nM) to doxorubicin and vincristine estimated by the microtitration assay and by clonogenic assay.

ID50 (nM)
39
31
42
35
27
34
31
32
38
20

Mean = 32.9 nM

Standard deviation = 6.332 nM

Co-efficient of variation = 19.3%

Table 5.4

Co-efficient of Variation for microtitre assay. The results for 10 separate estimations of the sensitivity of cell line SK-MES to exposure to doxorubicin (ID50, nM) for 24 hours are shown.

**Doxorubicin Penetration and MTT-Formazan Production in Aggregates
from Newly Established SCLC Cell Lines**

The distribution of doxorubicin and of MTT-formazan in sections of small cell lung cancer cell line aggregates are shown in Plate 5.1 (LS111) and Plate 5.2 (LCPH3). The aggregates were first observed by light microscopy (A). Doxorubicin fluorescence was observed throughout the aggregates (B). Similarly MTT-formazan crystals were seen to have been produced by all the cells throughout the aggregates (C). The only exception observed was for the larger aggregates of LS111. Direct examination of sections from such aggregates indicated that the viable cells appeared to be located in the outer layers of the aggregate. This viable cell area corresponded to the region of doxorubicin fluorescence and MTT-formazan production. A few cells in the centre of these larger aggregates showed doxorubicin fluorescence and similarly a few cells in the centre of the aggregates were associated with MTT-formazan production.

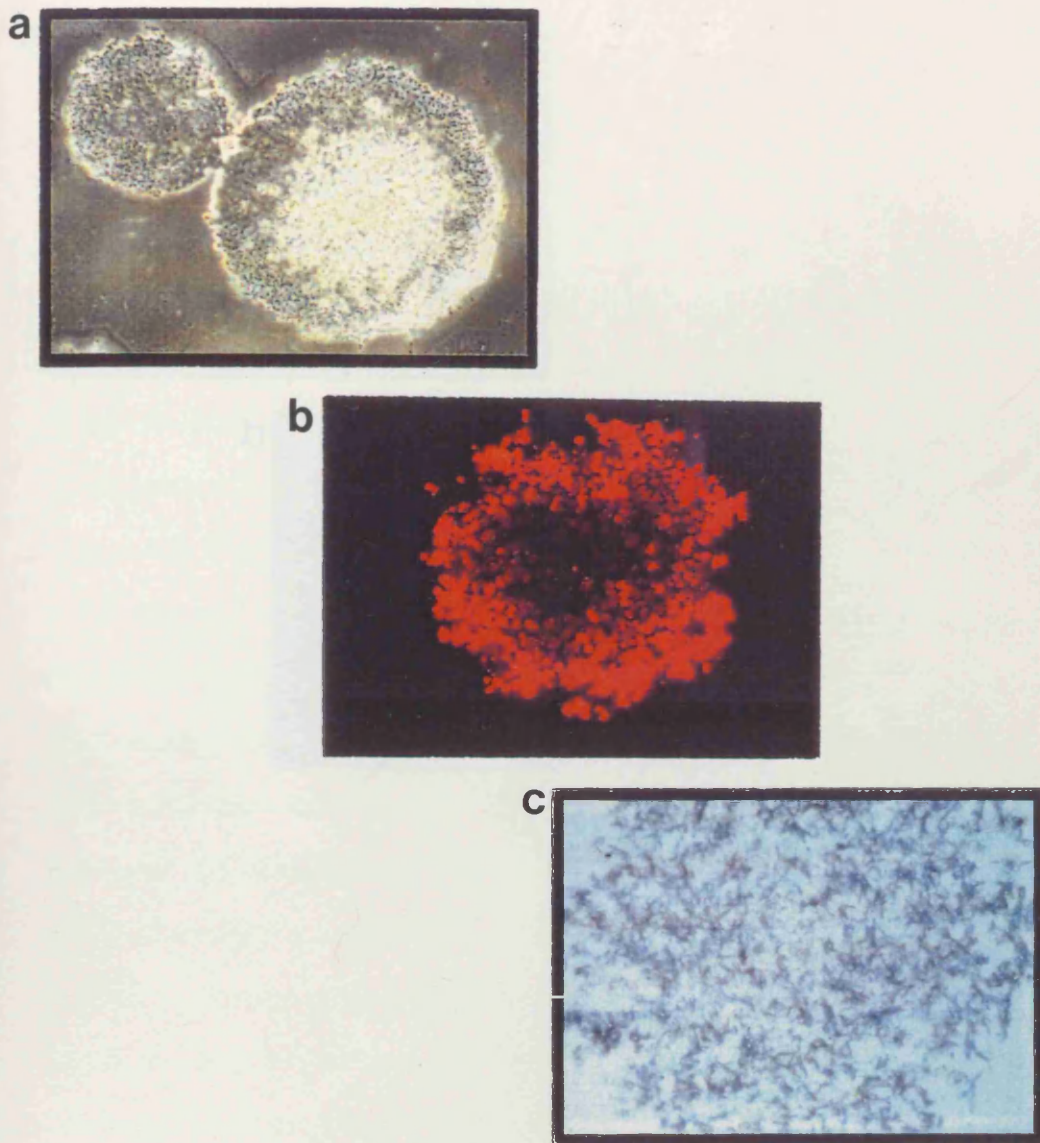


Plate 5.1

Penetration of doxorubicin into and MTT-formazan production by SCLC tumour aggregates from cell line LS111. For doxorubicin penetration studies cells were incubated with doxorubicin (10^{-6} M) for 4 hours. For MTT-formazan production studies cells were incubated with MTT (5mg/ml) for 4 hours. Frozen sections were prepared and observed unmounted to assess the viable cell population^(a). Subsequently sections were mounted in "UV inert". Those aggregates incubated with doxorubicin were viewed by fluorescent microscopy^(b). Those incubated with MTT were examined by light microscopy^(c).

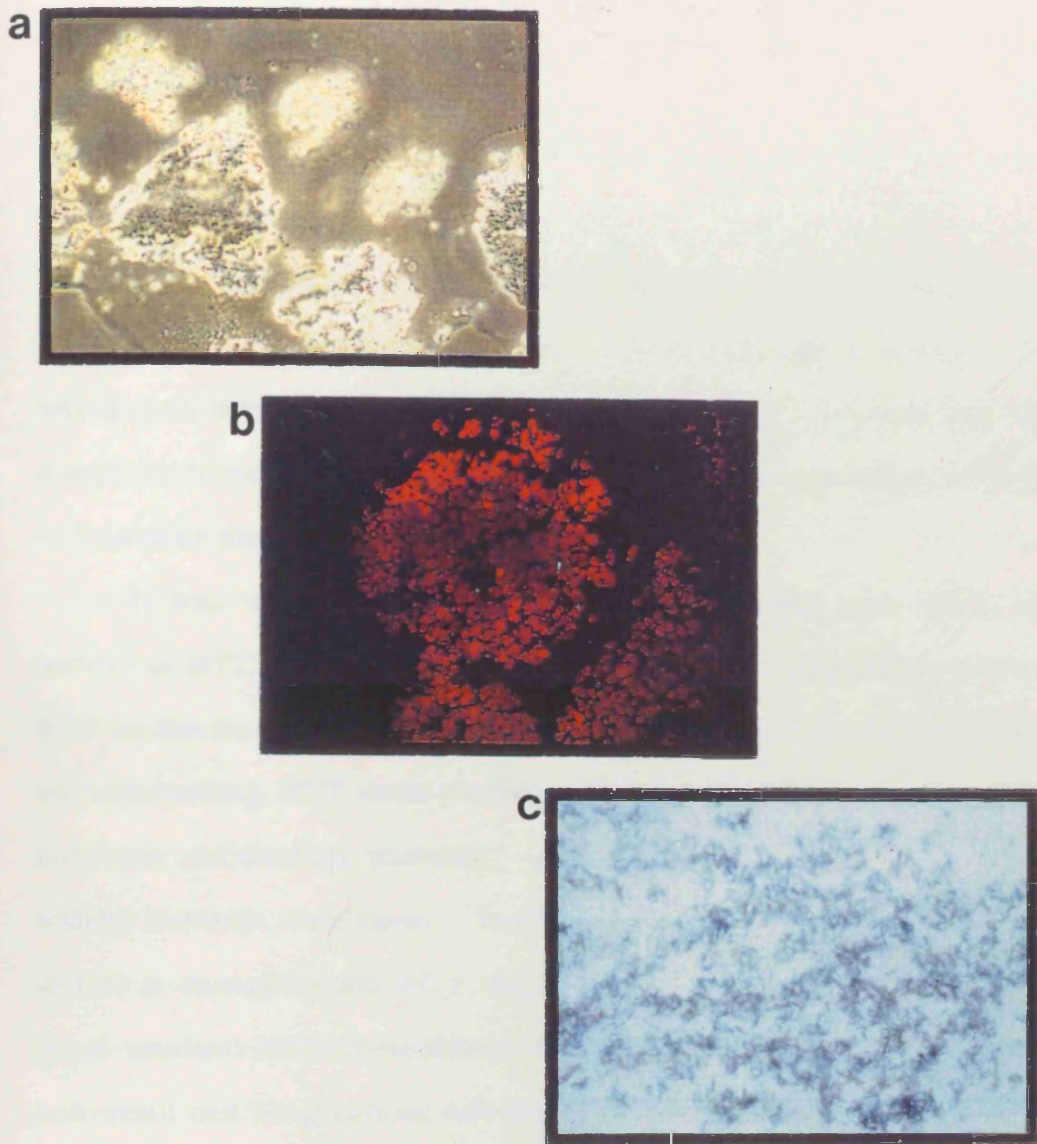


Plate 5.2

Penetration of doxorubicin into and MTT-formazan production by SCLC tumour aggregates from cell line LCPH3. For doxorubicin penetration studies cells were incubated with doxorubicin (10^{-6} M) for 4 hours. For MTT-formazan production studies cells were incubated with MTT (5mg/ml) for 4 hours. Frozen sections were prepared and observed unmounted to assess the viable cell population^(a). Subsequently sections were mounted in "UV inert". Those aggregates incubated with doxorubicin were viewed by fluorescent microscopy^(b). Those incubated with MTT were examined by light microscopy^(c).

DISCUSSION

The end point of any chemosensitivity assay is usually an estimate, either direct or indirect, of surviving cell numbers. The tetrazolium dye assay relies on the ability of cells to reduce dye in a quantitative manner and for there to be a linear relationship between cell number and dye reduction.

It was shown that when cells are incubated with MTT, the amount of MTT-formazan produced depends upon the concentration of MTT in the incubation medium. MTT-formazan production increases with increasing MTT until a concentration is reached at which MTT-formazan production plateaus. This optimum concentration varies widely between cell lines. In the SCLC cell lines (NCI-H187 and GLC4) a concentration of 2 mg/ml (final concentration 0.4 mg/ml) gives maximal MTT-formazan production. However, for some of the non-small cell lung cancer cell lines a concentration of up to 5 mg/ml (final concentration 1 mg/ml) is required for maximal absorbance readings.

The concentration of MTT used in previous assays has varied widely. Indeed, the original assay as described by Mosmann (1983) and the more recently reported screening assay (Carmichael et al., 1987) use final MTT concentrations of only 0.4 mg/ml. From the results of this study it appears that this would be insufficient for maximal formazan production in all cell lines. Denizot & Lang (1986) reported that in a T-cell lymphoma cell line, a final MTT concentration

of 1 mg/ml was required for maximal formazan production. The failure to determine the optimal concentration of MTT required may, in part, account for the failure of previous attempts using tetrazolium dye reduction to demonstrate a linear relationship between the amount of dye reduced and cell number (Carmichael et al., 1987; Cole, 1986; Mosmann, 1983).

With regard to the duration of incubation with MTT it is important to allow sufficient time for maximal reduction of MTT to MTT-F. The majority of workers have used a defined 4 hour incubation (Carmichael et al., 1987, 1988A; Green et al., 1984B; Mosmann, 1983; Ruben & Neubauer, 1987) although Cole (1986) used only a 3 hour incubation. Twentyman & Luscombe (1987) showed that MTT-F continues to be produced for up to 6 hours, although the vast majority has appeared within 4 hours. Similarly in the non-small cell lung cancer cell lines, MTT-F production appeared to plateau after 3 hours (Figure 5.4). Thus 4 hours appears sufficient time to allow most of the tetrazolium dye to be reduced, and hence was chosen as the defined incubation time for this assay.

Using the appropriate concentration of MTT for the cell line being studied and an appropriate incubation period, a linear relationship between cell number and dye reduction was demonstrated, up to a cell number of 2×10^4 cells per well (Figure 5.5). However, when the regression line is extrapolated it does not pass through the origin. Indeed in Figure 5.5 it appears that MTT-formazan is produced in the absence of any cells. Since both medium and MTT

are used to establish background absorbance values for reading the plate this observation suggests that the relationship may not be linear. It is interesting to note that although Cole (1986) has claimed a linear relationship for the assay, a curve would clearly be a better fit (see Figure 1, Cole, 1986).

To try and explain this non-linearity the spectral analysis of MTT and MTT-formazan produced commercially as well as produced by cells at low and high cell density was studied. Commercially prepared MTT-formazan when dissolved in DMSO is not the same colour as that produced by incubation of cells in the presence of MTT. This observation was confirmed by spectral analysis of the two MTT-formazans. Commercially prepared MTT-formazan exhibits a major absorption maximum at a wavelength of 510 nm and a shoulder in the peak which may represent a second absorption maximum at 560-580 nm. On the other hand, MTT-formazan produced by cells (4000 cells per well) exhibits a single absorption maximum at 560-570 nm (Figures 5.2 and 5.6). This difference has been reported previously (Alley et al., 1988) and has been attributed to the presence of serum when MTT-formazan from cells is dissolved in DMSO. This can not be the explanation because it was shown that at high cell density (10^4 /well) the absorption spectrum of the MTT-formazan product resembles that of the commercially prepared MTT-formazan, despite the presence of contaminating serum.

Nonetheless medium does interfere with the measurement of absorbance (Table 5.1). If a small volume (5-10 μ l) of medium is

added to a solution of MTT-formazan there is a marked increase in the absorbance readings. On the other hand if larger volumes of medium (20 ul - 50 ul) are added there is only a modest increase in absorbance values. Although it has been suggested that this effect is due to the protein present in the medium it is not clear why a small amount of protein would increase the absorbance more than a larger amount.

It is, however, known that pH can influence absorption spectra. Denizot & Lang (1986) suggested that the formazan absorption spectrum may be shifted by changes in pH. At an acid pH (3.5) the absorption spectrum of cellular MTT-formazan resembles that of commercially prepared MTT-formazan in DMSO alone and thus the absorbance measured at 570 nm is low. However, if the pH of the solubilised cellular MTT-formazan is increased, the spectrum approaches that of the MTT-formazan produced by cells at low density (4,000 cells per well, Figure 5.6) and thus the absorbance at 570 nm is markedly increased. This increase in absorbance at 570 nm with increasing pH reaches a plateau value at a pH of 10.5, when the MTT-formazan absorption spectrum exhibits only a single peak, at 570 nm (Figure 5.7).

pH Dependence of the MTT Assay

It is difficult to explain the pH dependence of the absorption spectrum of MTT-formazan. When MTT is reduced, the tetrazolium

ring is opened and the quaternary amine is reduced to a tertiary amine. A second tertiary amine (which originally formed one of the bonds to the quaternary amine) therefore becomes bonded to a hydrogen atom. It could be hypothesised that at a high pH this hydrogen is removed and so the tertiary amine is further reduced to a secondary amine with a lone pair of electrons which could delocalise to give a resonance form of MTT-formazan. Hence this resonance form would account for the absorption maximum at 570 nm, whereas the species in which the hydrogen ion is present as part of a tertiary amine might account for the absorption maximum at 510 nm. Whatever the explanation, these spectral shifts produce a real problem when dye reduction is used to estimate cell numbers. At low cell numbers the absorption maximum is at 560 - 570 nm whereas at high cell numbers there are two peaks; the larger at 510 nm and a smaller peak at 570 nm. Thus measurement of absorption at a single wavelength is inappropriate. To try and overcome the problem of spectral shift with varying pH, the pH of the MTT-formazan product was raised to a pH of 10.5. At this pH a single peak at 560 - 570 nm is observed regardless of cell number or the presence of "contaminating" culture medium. It was found that addition of a small quantity of buffer at pH 10.5 to the solubilised MTT-formazan product resulted in a significant increase in absorbance readings at 570 nm for wells with a high cell density. For example, a 50% increase was seen in the absorbance reading for A549 cells at a density of 3×10^4 cells per well (Figure 5.8). Furthermore it can be seen from Figure 5.8 that

addition of buffer (pH 10.5) resulted in a clear linear relationship between cell number and MTT-formazan production up to cell numbers of 4×10^4 per well and absorbance readings of 1.5. This range is well within the limits required for a chemosensitivity assay, even for cell lines with rapid doubling times. It is interesting to note that a strong base has previously been used to enhance the sensitivity of the nitroblue tetrazolium test but in this instance it was claimed that the base was used to increase the solubility of the formazan product in DMSO (Rook et al., 1985).

The importance of these observations is obvious when MTT reduction is used to estimate cell numbers in a chemosensitivity assay. If the MTT-formazan crystals are dissolved in DMSO alone, then the control cell number will be markedly underestimated and so the ID_{50} overestimated and thus is not similar to the ID_{50} obtained by a standard clonogenic assay. On the other hand, addition of buffer (pH 10.5) to these same wells results in a significant increase in absorbance (i.e. estimated cell number) in the control wells and in wells exposed to low concentrations of doxorubicin, such that the apparent ID_{50} is similar to that obtained by the clonogenic assay (Figure 5.11, Table 5.3).

Another important aspect of the dependence of the MTT-formazan absorption spectrum on pH relates to the effects of culture medium on absorbance readings. It was observed that MTT-formazan absorption is greatly increased in the presence of 10 μ l or less of culture medium but is only slightly increased in the presence of 10 μ l

to 40 μ l of medium. This phenomenon has been reported previously (Twentyman & Luscombe, 1987). However, addition of buffer at pH 10.5 causes a significant increase in the absorbance of MTT-formazan, and overcomes most of the variability in absorbance readings caused by the presence of culture medium (Table 5.1). This is an important observation, especially for chemosensitivity testing in non-adherent cell lines. With such lines it is impossible to remove all of the culture medium from the wells prior to the addition of DMSO. Thus if buffer is not added a significant source of error could result. In this regard it is interesting to note that Carmichael et al. (1987) reported a greater variability between wells for non-adherent cell lines in their screening assay, and they suggested that residual medium in the wells might have been responsible. No increase in variability with non-adherent cell lines was noted in this study and this could be explained by the inclusion of buffer at the final stage of the assay. It was also found that by controlling the pH with the incorporation of HEPES buffer during the MTT incubation period, the variability in MTT-formazan production between wells was decreased (Figure 5.9).

Chemosensitivity Testing

Another important aspect of the modified assay is the defined drug exposure period. This is clearly an arbitrary period. One requires long enough exposure to the toxic agent for cellular damage to occur (Twentyman, 1980). Cole (1986) showed that the apparent

sensitivity of cells was increased after a 24 hour exposure compared to a 1 hour exposure. However, a 72 hour exposure (without refeeding with drug at 24 and 48 hours) did not cause a further increase in sensitivity. Hamburger (1981) alluded to the problem of calculating an appropriate drug dosage in vitro (concentration and exposure time) to reflect the situation in vivo. Indeed she pointed out that tests in vitro do not take into account differences in host conversion and detoxification or tissue specific accumulation. Given these considerations a 24-72 hour exposure was felt most analagous to the situation in vivo. Although Ruben & Neubauer (1987) defined a 72 hour continuous drug exposure and Carmichael et al. (1987) used a 4 day continuous drug exposure, this length of exposure, if coupled with an appropriate recovery period (see below), would make the assay unnecessarily long.

Although a 24 hour drug exposure was used, the period could be varied to suit alternative experimental protocols. Previous reports which described tetrazolium based assays designed to be used in drug screening programmes have emphasised the importance of speed and automation (Alley et al., 1988; Carmichael et al., 1987). Because of this, these assays have included a continuous drug exposure for a period of up to 11 days. In one a limit of 7 days was chosen in order to avoid the need to feed cells during the assay (Carmichael et al., 1987). Thus the drug exposure time and growth period were combined.

The disadvantage of continuous drug exposure, relates to the

fact that it takes no account of drug stability or of drug metabolism, the products of which may be either active or inactive. The half-life of some mitomycins in tissue culture medium is about 2 hours and that of doxorubicin is only about 15 hours (Beijnen et al., 1986). As most of these chemosensitivity studies have been based on the effects of doxorubicin this may be relevant. It has been suggested that doxorubicin may alter the activities of mitochondrial enzymes, and so cause cardiac toxicity (Praet et al., 1986). Although the exact site or sites of MTT reduction in intact cells remain(s) to be elucidated, it is known that mitochondrial dehydrogenase activities can reduce the dye (Hess & Pearse, 1963; Slater et al., 1963). Thus with continuous drug exposure any residual doxorubicin could interfere with MTT reduction during the incubation period as a result of non-lethal damage to the cells. Furthermore, the absorption maximum for doxorubicin is between 500 nm and 600 nm and so any residual doxorubicin may contribute to absorbance measurements at 570 nm, as has been reported previously (Carmichael et al., 1987). For these reasons therefore a defined drug exposure time with a drug-free recovery period was used.

For studies of drug sensitivity, particularly where small changes in sensitivity are critical, a growth period is essential. It allows for elimination of cells killed by drug and distinguishes clearly between those cells which survive drug treatment but can no longer proliferate and those which continue to proliferate. The importance of this is demonstrated by a decrease in the ID_{50} over the first 3

days after drug exposure (Table 5.2). Another advantage is that differences in cell survival at different drug concentrations are amplified. This effect is maximised if the cells are fed daily so that growth is not limited by nutrient supply.

Previous work has shown that predictions of chemosensitivity based on observations of less than one cell cycle, both in HeLa cells (Freshney et al., 1975) and in glioma cells (Morgan & Freshney, 1979) can be misleading. Indeed some drugs may cause death only after several cell cycles (Morgan et al., 1983). Weisenthal et al. (1983A) have shown that cell survival assessed by dye exclusion in leukaemia cells decreased over a period of 6 days after a 1 hour exposure to doxorubicin. Thus it is important to allow sufficient time for damage to be manifest and for the control cells to replicate exponentially.

It has been stated that some workers used non-clonogenic viability tests too soon after noxious insult. Bhuyan et al., (1976) measured viability by dye exclusion immediately after drug exposure, and this would certainly not allow sufficient time for membrane function to be disturbed (Weisenthal & Lippman, 1985). On the other hand it is important to assay before cell division in the control wells has become contact inhibited (Ruben & Neubauer, 1987). Thus the time of assessment of viability after drug exposure may profoundly affect the results obtained. This time schedule dependency has also been described in the clonogenic assay system (Barranco & Townsend, Jr., 1986; Matsushima et al., 1985). A 3-day recovery period (approximately 3 cell doubling times), with daily feeding to ensure

cells were in exponential growth, was therefore used when this assay was applied to rapidly growing cell lines.

In terms of analysis of the data the ID_{50} was specified as the parameter for comparative purposes - both for comparing the sensitivity in different cell lines and also for studying the effects of verapamil on chemosensitivity (Chapter 6). The ID_{50} lies on the steep linear portion of the classic sigmoidal drug dose-response curve of log drug concentration versus response. Therefore the ID_{50} allows a reasonable estimation of drug potency with a limited number of drug concentrations and so provides a point that is easily reproducible (Ruben & Neubauer, 1987).

Thus, although the modifications made to the tetrazolium assay detract somewhat from its ability to be automated it appears suitable for studying the growth and chemosensitivity of the newly established SCLC cell lines. This was confirmed by demonstrating that doxorubicin penetrated into and MTT-formazan was produced by all the viable cells throughout the tumour aggregates (Plates 5.1 and 5.2). Because of the difficulties encountered in attempts to obtain an even distribution of aggregates from the newly established SCLC cell lines across the 96 well plate, individual aggregates were transferred by pipette into each well. This process was time-consuming but resulted in small inter-well variability in absorbance values.

Solvents Used

Although a number of workers have reported light scatter interference caused by an opaque alcohol-induced protein precipitate which forms in the presence of serum concentrations of greater than 10%-15% (Denizot & Lang, 1986; Green et al., 1984B) this was not a problem in these studies as medium containing serum concentrations greater than 10% was not used and DMSO and not an alcohol was used in the solubilisation step of the assay.

The solvents used to solubilise the MTT-F product have varied widely. The original paper by Mosmann (1983) described the use of isopropanol which had been acidified with hydrochloric acid to acidify the phenol red indicator in the culture medium (RPMI) to minimize interference with absorbance readings. Acidified isopropanol was also used by Green et al. (1984B) and Ruben & Neubauer (1987). However acid isopropanol has been found to be slow to dissolve the formazan crystals (Twentyman & Luscombe, 1987). Furthermore it may cause precipitation of protein in the medium resulting in high background absorbance values (Twentyman & Luscombe, 1987).

Alternative agents investigated include ethanol (Denizot & Lang, 1986) and mineral oil (Carmichael et al., 1987; Twentyman & Luscombe, 1987). However, mineral oil is not suitable as it again is slow to dissolve the formazan crystals and interferes with absorbance readings (Twentyman & Luscombe, 1987). This is particularly important when studying cell lines which grow as floating aggregates,

as in such cases there will inevitably be a small volume of medium remaining.

For these reasons the most widely used solvent is dimethyl sulphoxide (DMSO). This solvent has now been adopted by the majority of workers (Alley et al., 1988; Carmichael et al., 1987; Scudiero et al., 1988; Twentyman & Luscombe, 1987). Therefore, DMSO was chosen as the solvent for this assay also.

Scudiero et al. (1988) have recently investigated the use of an alternative tetrazolium dye (XTT) that is reduced to a water soluble formazan product thus eliminating the need for a solvent. However, XTT is metabolised much less efficiently than MTT and therefore a catalyst, phenazine methosulphate (PMS), is added during the assay. Moreover, this technique sometimes results in crystal formation and therefore requires that each well is individually examined. Furthermore PMS was found to cause non-specific deposition of formazan. Thus XTT is not a suitable alternative to MTT.

Limitations of the Assay

This assay, like many other non-clonogenic assays, is not without inherent limitations. It can not distinguish between cytostatic and cytocidal effects (Carmichael et al., 1987; Ruben & Neubauer, 1987). Thus it is unable to distinguish between cells within a population and the degree of response of each cell; for example, 50% inhibition of all cells and 100% inhibition (i.e. death) of 50% of cells

would give the same result. By incorporating a defined cell growth ("recovery") period in the absence of cytotoxic drug it has, in part, been possible to circumvent this problem (Ruben & Neubauer, 1987). Nonetheless the problem that the MTT assay can not quantify individual cell response remains.

Although this assay has not specifically been compared in detail with many of the available alternative assays, a good correlation of absorbance measurement with both cell counting and cloning was found. Previous workers have addressed this comparison in greater detail, in earlier work to validate the tetrazolium assay. For example, Heeg et al. (1985) compared the MTT assay with ^3H -thymidine uptake using Interleukin-2 producing helper T-cells. More recently Carmichael et al. (1987) used both a clonogenic assay and the dye exclusion assay described by Weisenthal et al. (1983B) to validate their tetrazolium based chemosensitivity assay.

Summary

A tetrazolium dye-based chemosensitivity assay which can be used for both adherent and non-adherent cell lines has been developed. The results obtained with this assay agree closely with those obtained by a standard clonogenic assay. Cells are exposed to drug for a defined period and then allowed to grow in drug-free

medium for 2 to 3 cell doubling times. MTT is used to assess surviving cell numbers and the MTT-formazan product is measured at a high pH so that all the MTT-formazan product is detected. This assay is also applicable to cell lines which grow as floating aggregates. Thus this assay may be used for studies of growth and chemosensitivity in the new SCLC cell lines in vitro. This work is presented in the next Chapter, along with studies of the activity of verapamil in the newly established SCLC cell lines.

CHAPTER 6

RESISTANCE MODULATION IN LUNG CANCER CELL LINES

INTRODUCTION

The tetrazolium-dye based assay has been modified and refined as described in the preceding Chapter. This assay is now suitable for the study of the growth and chemosensitivity of the newly established SCLC cell lines. The aim of this study was to determine the sensitivity of the cell lines to doxorubicin and to examine whether chemosensitivity in vitro reflects either the clinical history of the patient from whose biopsy material the cell line was established or the morphology or other characteristics of the cell line. In addition the activity of verapamil in the new SCLC cell lines was investigated to determine whether it has any effect on the sensitivity of these laboratory models of small cell lung cancer.

Verapamil is a calcium antagonist and is known to have significant effects on membrane ion flux (Kohlhardt et al., 1972). Verapamil modulates the slow calcium-dependent inward current systems and is used in the clinical setting to treat supraventricular tachydysrhythmias (Waxman et al., 1981) and angina pectoris (Simoons et al., 1980). As previously described (Chapter 1) it has also been shown to reverse drug resistance in vitro in a number of cell types (Beck et al., 1986; Slater et al., 1982; Tsuruo et al., 1982). Because of this attempts have been made to

use verapamil in combination with chemotherapy in cancer patients (Benson III et al., 1985; Dalton et al., 1989; Ozols et al., 1987A). However, use in patients is accompanied by dose limiting hypotension and heart block (Ozols et al., 1987A; Singh et al., 1978). As a result plasma levels achieved in patients are between 3 and 12 times lower than the most effective concentration in vitro (Benson III et al., 1985; Dalton et al., 1989; Kerr et al., 1986). For example, pharmacokinetic studies have shown that after an acute oral dose a peak verapamil plasma concentration of up to 1000 ng/ml (3 μ M) can be achieved. However in daily dosing or with an intra-venous infusion, the sustained plasma concentration achieved is only 200-500 ng/ml (0.6-1.5 μ M) (Benson III et al., 1985; Reiter et al., 1982, Tartaglione et al., 1983). Similar levels have also been reported by Frishman et al., (1982) and Hecht et al., (1981). Thus the sustained plasma levels of verapamil achievable in the clinic (0.6-1.5 μ M) are significantly lower than those that are known to be optimal in terms of resistance modifying activity in vitro (6 μ M). This problem has recently been reviewed by Chauffert and colleagues (1987).

Verapamil is a racemic mixture of the D- and L- stereoisomers. It is known that the L-isomer of verapamil is about 10 times more effective as a calcium antagonist than the D-isomer (Ferry et al., 1985). However the two isomers are equally potent blockers of the fast inward channel (Newrath et al., 1981). Since the effects of verapamil on drug resistance do not appear to be related to calcium antagonism (Ramu et al., 1984) it is conceivable that the D-isomer alone would be a more suitable agent to use

clinically, in view of the potential reduction in cardiovascular side-effects compared to the racemic mixture.

The resistance modifying activity of D-verapamil was therefore studied in an MDR SCLC cell line made resistant to doxorubicin and known to show a 10-fold increase in sensitivity to doxorubicin in the presence of racemic verapamil (6.6 μ M). Similarly, the effect of pre-treatment as a possible way of increasing the resistance modifying activity of verapamil was investigated. Finally the use of other agents (quinidine and bepridil) as resistance modifiers was evaluated in a range of lung cancer cell lines. Previous work has suggested that these agents may be used in the clinic at levels which are maximally active in terms of resistance modification in vitro without excessive toxicity (Tsuruo et al., 1984).

METHODS

Cell Lines

The newly established small cell lung cancer cell lines (LS106, LS111, LS112, LCPH2, LCPH3, LS263, LS274, LS277 and LS310) and their growth requirements have already been described. In addition a long established SCLC cell line and its resistant counterpart were also used. NCI-H69 was obtained from Dr. D. Carney (National Cancer Institute, Bethesda, U.S.A.) and its doxorubicin resistant counterpart H69LX10 was a gift from Dr. Peter Twentyman (M.R.C. Clinical Oncology and Radiotherapeutics Unit, Cambridge, U.K.). These lines grow in suspension and are maintained in RPMI 1640 medium containing glutamine (2mM), foetal bovine serum (10% v/v) and for H69LX10 only, doxorubicin (1 ug/ml, 1.84 uM).

In addition four long established non-small cell lung cancer (NSCLC) cell lines were used in some modifier experiments. The source and growth requirements of WIL, SK-MES, L-DAN and A549 have been described previously (Chapter 5).

Reagents/Drugs

Racemic verapamil (V), quinidine (Q), bepridil (B) and 3-(4,5-dimethylthiazol-2-yl) - 2,5-diaphenyltetrazolium bromide (MTT) were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). The stereoisomers of verapamil were a gift from Dr E.

Schlick (Knoll A.G., 6700 Ludwigshafen, Federal Republic of Germany).

Determination of Optimal MTT Concentration

Individual SCLC aggregates in 200 μ l of medium were placed in wells, 1 aggregate per round bottomed well of a 96 well microtitre plate (Nunc; Gibco, Paisley, Scotland) using a Gilson micropipette. For cell line LS112ST, exponentially growing cells were trypsinized and diluted to give a final concentration of 5×10^4 cells/ml. Aliquots, 200 μ l, were then placed in each well of a 96 well microtitre plate (flat bottomed; Linbro, Flow Laboratories). Cells were incubated for 24 hours at 37°C in an atmosphere of 2% CO₂ in air. Subsequently MTT (at concentrations of between 0 and 5 mg/ml) was dissolved in PBS and 50 μ l was added to each well, 8 wells per concentration. Following a 4 hour incubation the plates were centrifuged at 200 g for 5 minutes and the medium was carefully removed. The formazan crystals produced were dissolved in 200 μ l DMSO and 25 μ l of Sorensen's glycine buffer (0.1M glycine + 0.1 M NaCl equilibrated to a pH of 10.5 with NaOH 0.1N) was added. Absorbance was recorded as described previously (Chapter 5).

Estimation of Growth Rates

The newly established SCLC cell lines were plated out into round bottomed 96 well plates (Nunc; Gibco, Paisley, Scotland),

one aggregate per well in 200 μ l of medium. In all cases duplicate plates were set up. Plates were fed daily with fresh medium. Every 2nd day the aggregates from one row (comprising eight wells) from each plate were transferred to another 96 well plate for measurement of MTT reduction. MTT (50 μ l, 4 mg/ml) was added and the plates processed as described in the preceding Chapter.

Cytotoxicity Assays

Drug sensitivity was determined by the tetrazolium dye-based microtitre assay as described in detail in Chapter 5. For the SCLC cell lines, 1 aggregate was transferred into each well in 100 μ l of medium using a Gilson micropipette. Freshly diluted drug (\pm modifier) was added immediately in a volume of 100 μ l at twice the desired final concentration. It should be noted that when verapamil, the stereoisomers of verapamil or other modifiers were used, a serial dilution of 8 concentrations of doxorubicin was freshly prepared at twice the desired final concentration. These were then diluted 50:50 with either drug-free medium or medium containing modifier at twice the desired final concentration (four times the desired final concentration for the new SCLC cell lines) prior to addition to the wells. Four wells (adherent cells) or 8 wells (non-adherent cells) were used for each drug concentration and when modifiers were used, the control wells were exposed to the modifier alone.

Results are expressed in terms of the drug concentration required to kill 50% of the cells (ID_{50}) estimated as the absorbance

value equal to 50% of that of the cells in the control, untreated wells.

RESULTS

Determination of Optimal Concentration of MTT

The optimal concentration of MTT for maximal formazan crystal production varied from 1.6 mg/ml (LS112ST, Figure 6.1A) to 4 mg/ml (LS111, Figure 6.1B). Concentrations of MTT up to 5 mg/ml did not appear to be significantly toxic to the new SCLC cell lines.

Growth Studies

Representative growth curves for two of the newly established cell lines [LS111 (A) and LS112FL (B)] are shown in Figure 6.2. The doubling times, measured from the growth curves, for all the newly established SCLC cell lines are shown in Table 6.1.

Determination of Chemosensitivities of Newly Established SCLC Cell Lines

The results of a chemosensitivity assay are illustrated in Plate 6.1 which shows a microtitre plate at the end of a chemosensitivity assay, following a 4 hour incubation with MTT (4 mg/ml) before (A) and after (B) solubilisation of the MTT-formazan crystals in DMSO. Figure 6.3 shows a representative dose response curve to doxorubicin for the newly established SCLC cell

LS112 ST

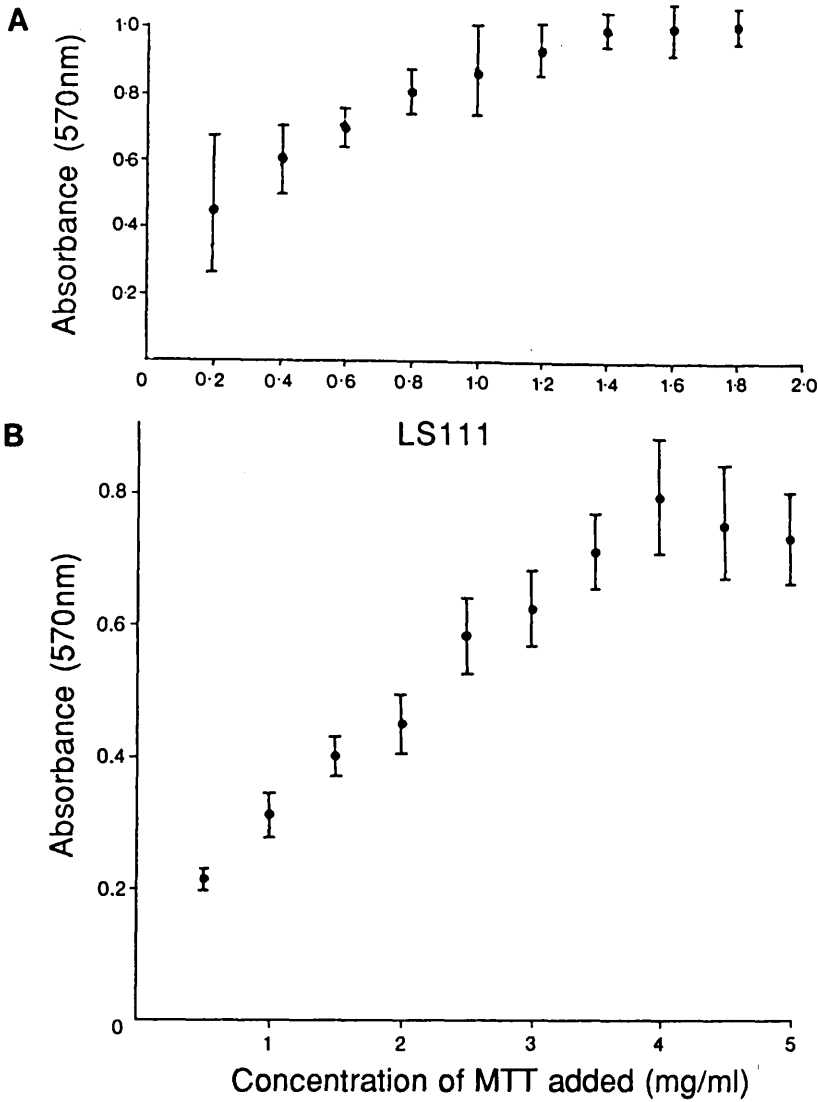


Figure 6.1

Determination of optimal MTT concentration in SCLC cell lines. For cell line LS112ST (A) cells were plated out (3×10^4 per well) and allowed to attach and grow for 2-3 days. For cell line LS111 (B) aggregates were placed in wells (1 aggregate per well) in 200 ul of culture medium and allowed to grow for 1-2 days. Subsequently 50ul MTT (concentration 0-5mg/ml) was added to each well, 8 wells per concentration. Plates were incubated for 4 hours and then MTT-formazan production measured, after the MTT-formazan crystals had been dissolved in DMSO. Values are the mean \pm SEM of 8 observations.

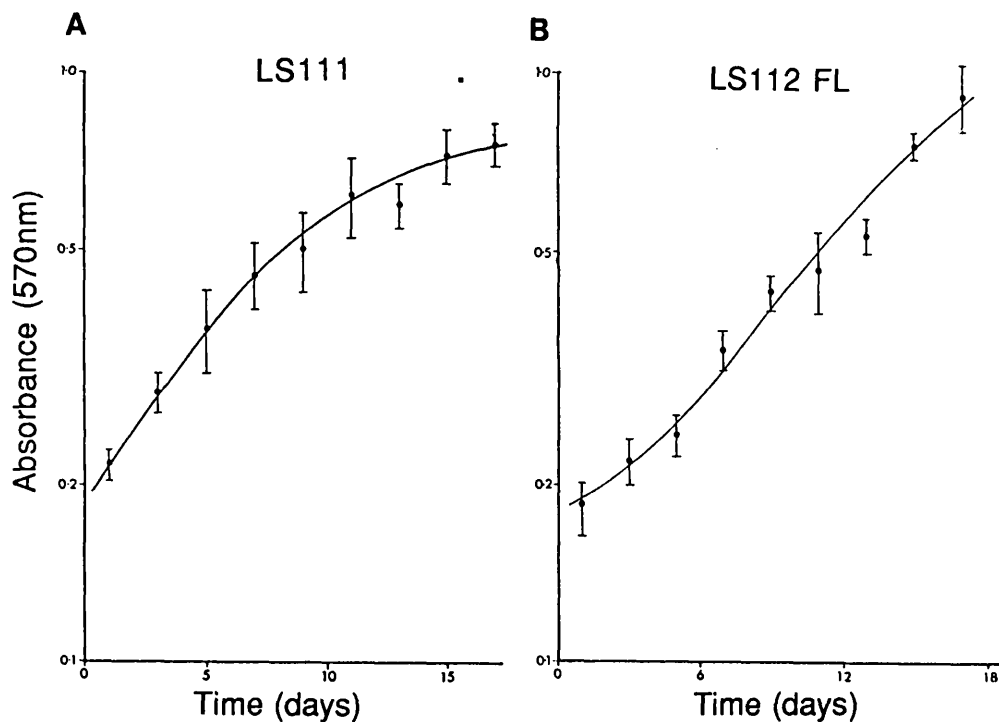


Figure 6.2

Growth curves for LS111 (A) and LS112FL (B). Individual aggregates were plated out in 200ul of medium in 96-well plates. The plates were fed daily and every second day aggregates from 8 wells (1 row) were transferred to another plate for measurement of viable cell number by MTT reduction. Cells were incubated with MTT (50ul, 4mg/ml) for 4 hours and the MTT-formazan crystals dissolved in DMSO. Values are the mean \pm SEM of 8 wells.

Cell Line	Doubling Times (Days)	ID50 (DOX, nM)
LS106	5.5	127.0* \pm 10.9 (3)
LS111	5.1	105.0 \pm 23.0 (4)
LS112FL	6.2	35.5 \pm 11.4 (4)
LCPH2	4.5	73.5 \pm 10.8 (4)
LCPH3	5.6	38.5 \pm 9.6 (4)
LS263	4.0	147.0 \pm 81.0 (3)
LS274	5.5	220.0 \pm 40.0 (6)
LS277	4.5	17.5 \pm 7.7 (3)
LS310	5.0	1050.0 \pm 140.0 (6)

* Mean \pm SEM

Table 6.1

Doubling times and chemosensitivities to doxorubicin (DOX) of newly established SCLC cell lines

The number of observations are shown in parentheses.

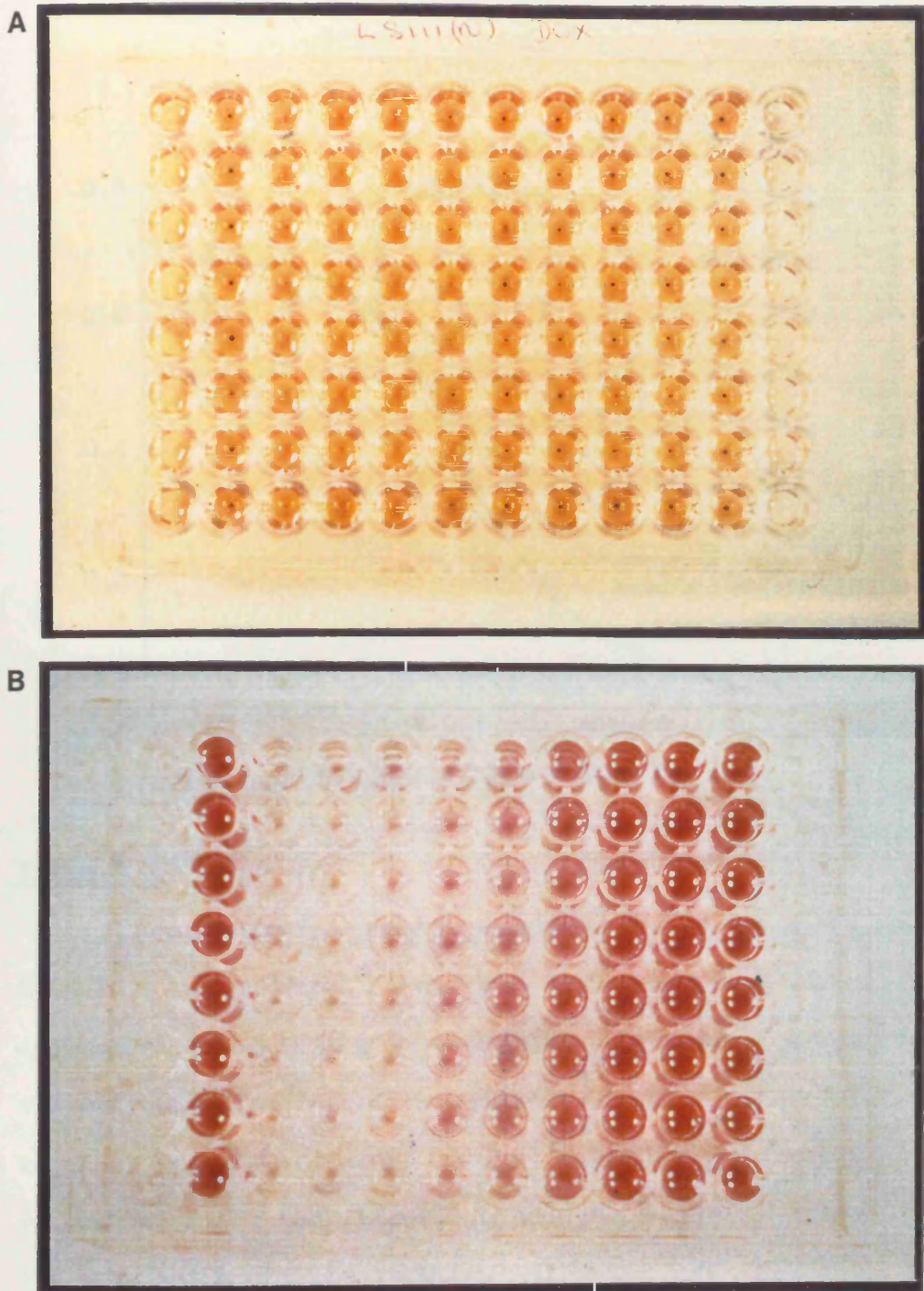


Plate 6.1

A 96-well plate at the end of a chemosensitivity assay, to determine the sensitivity of cell line LS111 to doxorubicin. The plate was incubated with MTT (50ul, 4mg/ml) for 4 hours and is shown before (A) and after (B) the MTT-formazan crystals were dissolved in DMSO.

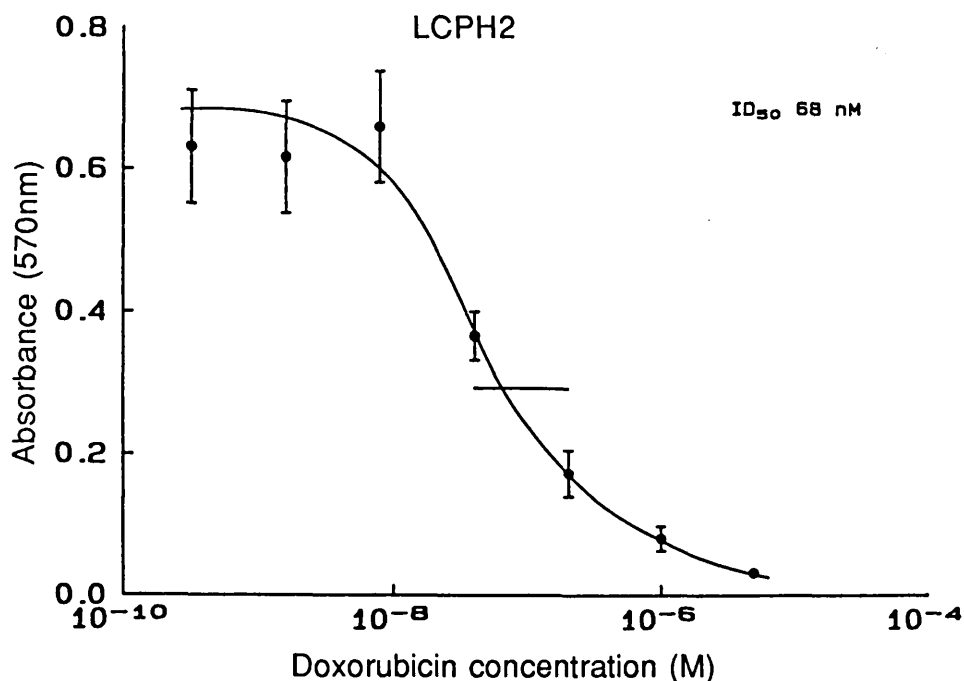


Figure 6.3

Chemosensitivity assay to determine sensitivity of cell line LCPH2 to doxorubicin. Individual aggregates were placed (1 aggregate per well) in a 96-well plate. Cells were exposed to various concentrations of doxorubicin for 24 hours. After a recovery period (with daily feeding) surviving cell number was estimated by MTT reduction (50ul, 4mg/ml, 4 hours). Values are the mean \pm SEM of 8 observations.

line LCPH2. Figure 6.4 shows the results of 4 separate experiments performed by 2 individual researchers to estimate the chemosensitivity of cell line LS111 to doxorubicin. As can be seen the experimental results are highly reproducible. The results for all the chemosensitivity assays in the newly established SCLC cell lines are shown in Table 6.1. It can be seen that the cell lines exhibit a wide (60-fold) range of sensitivity to doxorubicin. The intrinsic chemosensitivity to doxorubicin did not appear to relate to the growth morphology or other characteristics of the cell line, nor did it relate to the patient history from whose biopsy the cell line was established.

Effects of Racemic Verapamil and its Stereoisomers on Drug Sensitivity in H69LX10

The sensitivity of the MDR SCLC cell line H69LX10 to doxorubicin is shown in Table 6.2. H69LX10 is about 60-fold more resistant than NCI-H69. Also shown in Table 6.2 is the sensitivity to doxorubicin when cells were exposed to drug in the presence of either racemic verapamil (6.6 μ M) or to the individual stereoisomers of verapamil (6.6 μ M). Racemic verapamil had no significant effect on the chemosensitivity of the parent cell line. However, verapamil increased the sensitivity to doxorubicin of the resistant cell line by 10-12 fold. Furthermore, this effect was observed with both the D- and L- isomers of verapamil.

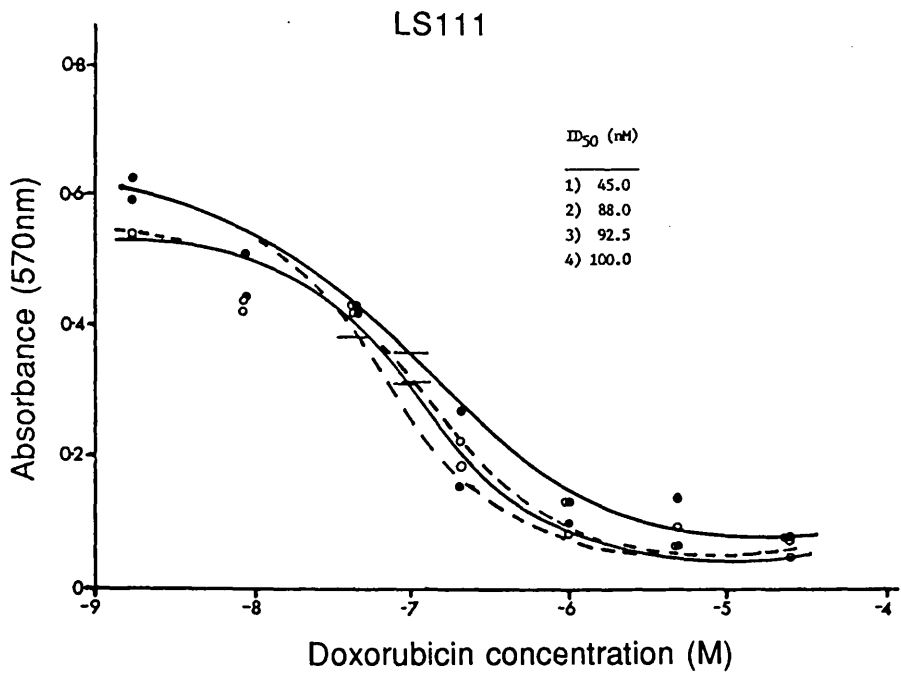


Figure 6.4

MTT-formazan production per well estimated 5 days after exposure of individual aggregates of cell line LS111 to various concentrations of doxorubicin for 24 hours. Results are the mean of 8 wells and 4 separate experiments are shown.

Cell Line	Modifier			
	DOX	DOX + V	DOX + DV	DOX + LV
H69 (ID50, nM)	45.0 \pm 7.0	44.0 \pm 6.2	-	-
H69LX10 (ID50, uM)	2.68 \pm 0.59	0.22 \pm 0.05	0.27 \pm 0.02	0.25 \pm 0.02

Table 6.2

The resistance modifying activity of the stereoisomers (DV, LV) of verapamil (V) in H69LX10 cells exposed to doxorubicin (DOX). Results are shown +/- SEM of 3 observations.

Concentration Dependent Effect of Verapamil

Figure 6.5 shows the chemosensitivity to doxorubicin when H69LX10 multi-drug resistant cells are exposed to the drug in the presence of various concentrations of either racemic verapamil or the D-isomer. There is a concentration-dependent increase in drug sensitivity in the presence of verapamil. This is true for both racemic verapamil and the D-isomer. There is no significant difference between the activities of the two agents at any given concentration. The maximum activity of verapamil is observed at a concentration of 6-7 μM .

Effect of Pre-treatment with Verapamil

The results of experiments in which cells were exposed to the resistance modifier verapamil alone for 24 hours, immediately prior to standard co-incubation with doxorubicin for 24 hours are shown in Table 6.3.

It can be seen that in the case of the cell line WIL, pre-treatment with verapamil for 24 hours leads to a further significant increase in resistance modification (i.e. increase in sensitivity to doxorubicin). On the other hand, there is no increase in resistance modification following pre-treatment of the resistant SCLC cell line H69LX10 with verapamil.

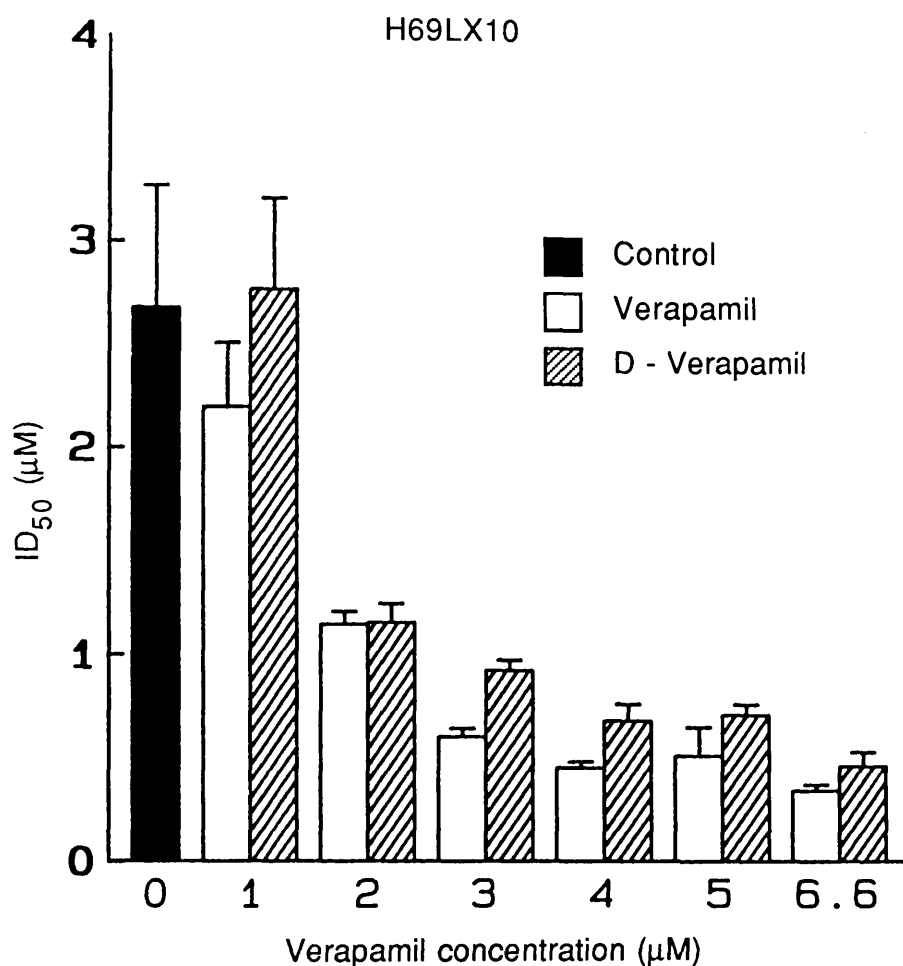


Figure 6.5

The effect of various concentrations of either racemic verapamil or the D-isomer, on the sensitivity of cell line H69LX10 to doxorubicin. Cells were exposed to doxorubicin for 24 hours in the absence (solid bar) or presence of racemic verapamil (open bars) or D-verapamil (hatched bars). The ID₅₀ is the drug concentration required to kill 50% of the cells, and results are the mean \pm SEM of 3 observations.

Cell Line	DOX	DOX + V	Pre-treatment
WIL (ID50, nM)	76.9 ± 3.9	21.7 ± 4.3 (3.5)	6.3 ± 0.7 (12.2)
H69LX10 (ID50, uM)	8.28 ± 1.10	0.69 ± 0.07 (12.1)	0.67 ± 0.04 (12.4)

Table 6.3

Effect of pre-treatment with verapamil (V) on the sensitivity of WIL and H69LX10 to doxorubicin (DOX). Results shown are the mean +/- SEM of 3 observations. The fold change in sensitivity in the presence of modulator is shown in parentheses.

Effects of Other Resistance Modifiers in H69LX10

Table 6.4 shows the effect of a number of resistance modifiers on the chemosensitivity of H69LX10 to doxorubicin. In this Table the effects of the modifier are shown by comparing the ID_{50} with doxorubicin alone to the ID_{50} achieved with doxorubicin plus modifier. This gives a ratio which may be regarded as a resistance modifying factor. It may be seen that bepridil at a concentration of 4 μ M (which can be achieved in the clinic) results in a 6-fold increase in sensitivity to doxorubicin. Quinidine is even more effective as a resistance modifier in this cell line, causing a 10-fold increase in sensitivity to doxorubicin at clinically achievable levels. Furthermore for both quinidine and bepridil there is clear evidence of a dose-response relationship.

Effects of Resistance Modifiers in Established NSCLC Cell Lines

Table 6.5 shows the effects of a number of resistance modifiers on the sensitivity of the NSCLC cell lines to doxorubicin. It may be seen that in these cell lines although verapamil (V) and the L-stereoisomer of verapamil (LV) do modulate chemosensitivity to doxorubicin significantly in all cases, the D-isomer (DV) is less effective and neither quinidine (Q) nor bepridil (B) have any modulating effect.

		ID50 DOX ID50 DOX + Modifier (6.6 μ M)				
Cell Line	ID50 (nM)	+V	+LV	+DV	+Q	+B
A549	23 \pm 4	9.1	12.5	4.4	1.4	1.1
L-DAN	23 \pm 4	2.7	2.9	2.3	1.0	1.2
SK-MES	33 \pm 2	3.5	-	-	0.8	0.6
WIL	77 \pm 4	4.5	5.3	1.7	0.9	1.4

Table 6.5

The activity of a range of resistance modifiers in NSCLC cell lines. Results are the mean \pm SEM of 3 observations and the ratio ID50 DOX/ID50 DOX + modifier gives an indication of the activity of each modifier in each cell line.

Effects of Verapamil in Newly Established SCLC Cell Lines

The effects of verapamil (6.6 μ M) on the sensitivity of the newly established SCLC cell lines to doxorubicin is shown in Table 6.6. Also shown in this Table are the growth characteristics of the cell lines and the history of the biopsies (pre-treatment or post-chemotherapy) from which the cell lines were established.

It can be seen that there was a significant increase in sensitivity to doxorubicin in the presence of verapamil in 5 of the 9 cell lines. This effect is illustrated in Figure 6.6 which shows a 2.3 fold increase in sensitivity to doxorubicin in the presence of verapamil (6.6 μ M) in cell line LS263. The modulation of sensitivity with verapamil did not appear to relate to the clinical history of the patient from whose biopsy the cell line was established, nor did it appear to relate to any particular characteristics of the cell line, including morphology. Nor did sensitisation by verapamil relate to the intrinsic sensitivity of the cell line to doxorubicin.

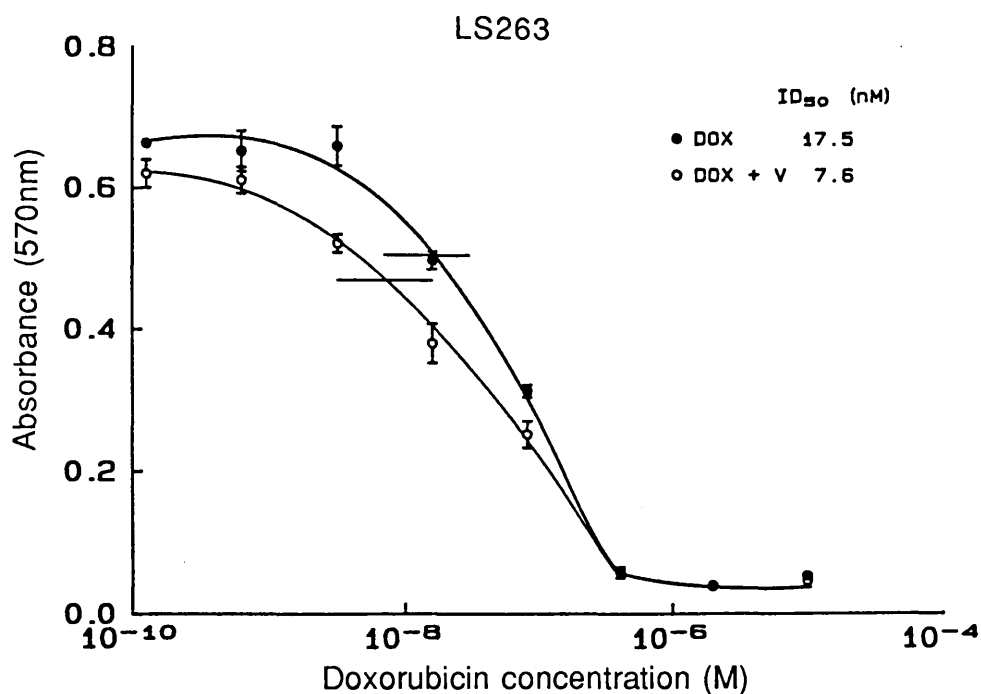


Figure 6.6

MTT-formazan production per well estimated 5 days after exposure of cell-line LS263 to doxorubicin alone for 24 hours (●) or to doxorubicin and verapamil (6.6uM) for 24 hours (○). When verapamil was used control wells were also exposed to verapamil (6.6uM) for 24 hours. Values are mean \pm SEM of 8 wells.

DISCUSSION

The results presented here are of substantial importance. They show that it is possible to accurately measure chemosensitivity in the newly established small cell lung cancer cell lines. A wide range of sensitivity to doxorubicin has been demonstrated. These results also indicate that verapamil has a modulating effect on the chemosensitivity of a number of lung cancer cell lines - not only in the non-small cell lung cancer cell lines (Table 6.5) and in the MDR SCLC cell line H69XL10 (Table 6.2) but also in a number of the newly established SCLC cell lines (Table 6.6).

Chemosensitivity of Newly Established SCLC Cell Lines

Having been able to accurately measure the growth and chemosensitivity of the newly established SCLC cell lines it has been possible to examine whether in vitro chemosensitivity reflects either the clinical history of the biopsy material from which the cell line was established or the morphology of the cell line.

These questions have intrigued scientists for many years and results remain conflicting. For example, Hill (1983) and Cole (1986) showed a correlation between the clinical response of certain tumour types and the response to cytotoxic drugs in vitro using established cell lines of similar histology. On the other hand, Hug et al., (1984) found no association between the chemosensitivity of cell lines obtained from breast cancer patients before and after

chemotherapy. More specifically in small cell lung cancer early work by Carney et al. (1983) suggested that cell lines established from untreated patients were relatively sensitive and cell lines established from relapse biopsies were relatively resistant. This work is, however, open to criticism. A cloning technique, with all the inherent problems of aggregate disruption described previously, was used to measure chemosensitivity. Furthermore the definition of sensitivity or resistance used by these researchers as a greater or less than 70% reduction in survival must be regarded as rather simplistic and arbitrary.

Work by Carmichael et al. (1988A) using a tetrazolium dye-based chemosensitivity assay, in a study of 15 newly established SCLC cell lines (7 from pre-treated patients and 8 from patients after chemotherapy) also suggested that small cell lung cancer cell lines established from treated patients were more resistant than cell lines from untreated patients. However, the chemosensitivity assay described by Carmichael et al. (1988A) required the disaggregation of the SCLC cell lines prior to seeding, and this may have led to inaccuracies.

Recently a group of workers in Holland have established 3 new small cell lung cancer cell lines from one patient, one cell line before treatment and two cell lines following relapse (Berendsen et al., 1988). All the cell lines showed similar morphology to the biopsies from which they were derived. Chemosensitivity was measured using Weisenthal's fast green assay. The relapse cell lines showed increased resistance to doxorubicin, melphalan and etoposide compared with the cell line from the original biopsy.

However, it is worth noting that the third cell line, established from a biopsy at a time of further relapse was actually more sensitive than the cell line established from the initial relapse tumour. The authors attribute this to alterations in chemosensitivity in vitro.

Carney et al. (1983) reported that SCLC cell lines of variant morphology were radio-resistant. It could be hypothesised that these findings with regard to the radio-resistance of variant lines may simply relate to the method used to study cell sensitivity. It seems likely that classic cell lines will require more aggressive physical disaggregation and hence will be more sensitive to the effects of noxious injury (e.g. radiation). On the other hand, more easily disaggregated variant morphology cell lines will be relatively protected from toxic injury and so appear more resistant if radio-sensitivity is measured by cloning. Similarly the apparent increased chemosensitivity of the SCLC cell line H69, in comparison to non-small cell lung cancer cell lines, reported by Cole (1986) may relate to disaggregation of H69 for counting prior to plating out in microtitre plates. Nonetheless this can only partly be the explanation because neither Carney et al. (1983) nor Carmichael et al. (1988A) showed a correlation between aggregate morphology and chemosensitivity.

In this study 9 cell lines, 5 from untreated patients and 4 from relapse patients, two of which have been passaged in mouse xenograft, have been established. Although a 60-fold range of chemosensitivity has been demonstrated no clear relationship

between clinical history of the biopsy or aggregate morphology and in vitro chemosensitivity has been found (Table 6.6). For example LS263 obtained from a relapse biopsy of a chemoresistant patient was almost twice as sensitive as LS274, a cell line established from a pre-therapy biopsy of a chemosensitive patient. However, it should be noted that, for an individual patient, chemosensitivity does appear to relate to patient/biopsy history and mirror clinical course. Thus LS310, a cell line obtained from a biopsy of a relapse patient is 5 times more resistant than cell line LS274, obtained from the pre-treatment biopsy of the same patient who initially had a chemosensitive tumour.

Resistance Modulation in NSCLC and an MDR SCLC Cell Lines

For the resistant cell line H69LX10 verapamil increased doxorubicin sensitivity and this effect was concentration dependent (Figure 6.5). Such an effect has been described previously for verapamil (Rogan et al., 1984) and also for amiodarone (Chauffert et al., 1987). In addition it was shown that the individual stereoisomers of verapamil are equally effective in terms of resistance modification (Table 6.2). These findings have implications for resistance modification in the clinic. It has already stated that levels of verapamil optimally active in vitro (6.6 μM) are not attainable in the clinic because of unacceptable cardiovascular toxicity. Given that the D-isomer of verapamil has been shown to be equally active in terms of resistance modification in the doxorubicin resistant cell line H69LX10, and given that D-

verapamil is thought to be less cardiotoxic (Ferry et al., 1985) it may be possible, with the use of D-verapamil alone, to achieve higher levels of modifier without excess cardiovascular toxicity. Although not maximal, it is worth noting that some resistance modification was seen in the resistant cell line H69LX10 with concentrations of verapamil that are achievable in the clinic (Figure 6.5).

An alternative approach to the use of the individual stereoisomers of verapamil in the clinic might be the use of other resistance modifying agents. Certainly it has been shown that the use of either bepridil or quinidine can produce a 6-fold or 10-fold increase in sensitivity to doxorubicin in H69LX10 cells (Table 6.4). Moreover this effect is seen with concentrations of modifier that are achievable in the clinic. Thus these agents may be more suitable for future clinical studies of resistance modification. However, it should be noted that neither bepridil nor quinidine, unlike verapamil, produce any increase in chemosensitivity in the non-small cell lung cancer cell lines (Table 6.5). Since these non-small cell lung cancer cell lines do not express P-glycoprotein this finding suggests that verapamil may have a different and/or additional mechanism of action as a modifier in these cell lines. It is interesting to note that the resistance modifying activity of verapamil in these cell lines appears to be related to the L- rather than the D-isomer (Table 6.5).

It has also been shown that pre-treatment for 24 hours with verapamil alone prior to cytotoxic drug exposure in the presence of verapamil can increase the resistance modification seen in the

non-small cell lung cancer cell line, WIL (Table 6.3). The reason for this remains unclear. A similar effect has previously been described by Radel et al. (1988) who showed that a 24 hour exposure to the modifier DMDP followed by a short exposure to doxorubicin resulted in the same cytotoxicity as a 24 hour exposure to both agents. Thus it may be that pre-treatment with modifier may be a method of obtaining increased resistance modification in the clinic. It should be noted that pre-treatment with verapamil did not increase the chemosensitivity of the resistant SCLC cell line H69LX10. This may be because with a 24 hour exposure to doxorubicin plus verapamil, the maximal resistance modification that could be obtained had already been achieved.

This work, like most of the work on resistance modifiers to date, has used cell lines derived from animal tumours or long established cell lines derived from human tumours. These cell lines have then been rendered resistant in vitro by chronic exposure to cytotoxic drugs, and used as models of tumour cell resistance. What is ideally required are new human cell lines established from patients whose tumour demonstrates the phenomenon of initial chemosensitivity and subsequent resistant relapse in the clinic. This underlines the importance of the newly established SCLC cell lines.

Resistance Modulation in Newly Established SCLC Cell Lines

The fact that 5 of the 9 newly established SCLC cell lines

studied show an increase in sensitivity to doxorubicin in the presence of verapamil is of particular note (Table 6.6). Such an effect has not been previously reported. This increase in chemosensitivity with verapamil did not relate to the intrinsic sensitivity of the cell lines to doxorubicin or to the clinical history of the patient from whose biopsy the cell line was obtained. For example, the most resistant cell line, LS310 obtained from a relapse biopsy from a chemoresistant patient did not show any change in sensitivity to doxorubicin in the presence of verapamil. On the other hand one of the most sensitive cell lines (LS112FL) established from a patient who had not received any treatment showed an almost 3-fold increase in sensitivity to doxorubicin in the presence of verapamil.

Thus verapamil can significantly modulate the sensitivity of some newly established SCLC cell lines. This suggests that verapamil may have a role in the clinical setting, and this has stimulated interest and enthusiasm for the randomised clinical trial of verapamil given in addition to chemotherapy to patients with small cell lung cancer. The results of this study are described in the next Chapter.

CHAPTER 7

A RANDOMISED CLINICAL STUDY OF VERAPAMIL IN ADDITION TO COMBINATION CHEMOTHERAPY IN SMALL CELL LUNG CANCER

INTRODUCTION

Verapamil has been shown to increase the drug sensitivity of MDR cells in vitro. This effect is known to be dose dependant. At optimal concentrations in vitro (6.6 uM), verapamil has also been shown to have modulating activity in 5 of the 9 newly established SCLC cell lines (Chapter 6).

Overgrowth with resistant tumour is the principle cause of treatment failure in SCLC. To date there have been few clinical studies, and no published large scale randomised clinical studies to examine the activity of resistance modifying drugs in the chemotherapy of cancer patients.

Previous Clinical Studies

The first study of verapamil given in addition to chemotherapy was that reported by Benson III et al. in 1985. Seventeen patients were treated with vinblastine (1.5 mg/m² for 5 days) and escalating doses of intravenous verapamil. Cardiovascular toxicity was the

dose-limiting toxicity for verapamil, and the mean verapamil level achieved was only 290 ng/ml (0.45 μ M). No response or survival data was reported from this study.

Ozol's group from the National Cancer Institute at Bethesda reported a study of verapamil given in addition to doxorubicin in the treatment of drug-resistant ovarian cancer (Ozols et al., 1987A). Eight patients were treated with doxorubicin (50 mg/m²) and increasing doses of intravenous verapamil (maximum dose 15 mg/kg/min). With this dosage a mean verapamil level of 1,273 ng/ml [2.0 μ M] (range 720 - 2,767 ng/ml [1.1 - 4.3 μ M]) was achieved. However, all patients required invasive cardiac monitoring and cardiovascular toxicity was unacceptable. There were no objective responses seen in any of the patients and median survival was only 4 months.

The few other studies exploring the efficacy of resistance modifiers have been slightly more encouraging. Presant et al. (1986) used verapamil (480 to 960 mgs/day orally) and observed some responses in heavily pre-treated patients. The higher doses of oral verapamil were noted to cause hypotension and arrhythmias. Dalton et al. (1989) have treated 8 patients with multiple myeloma and non-Hodgkin's lymphoma who had developed progressive disease whilst receiving chemotherapy with vincristine and doxorubicin. They treated these patients with the same chemotherapy and added infused verapamil (0.15 mg/kg/hour) for 36 hours. Three patients received higher doses of verapamil (0.30-0.45 mg/kg/hour). As in previous

studies, the dose limiting toxicity was cardiac. Three patients, two of whom had been treated with the higher doses of verapamil, showed a response, and these three patients were all shown to have P-glycoprotein positive tumours. As vincristine is known to increase intracellular anthracycline accumulation in MDR cells (Willingham et al., 1986), the addition of this cytotoxic drug may have potentiated the effects of verapamil on doxorubicin accumulation. However, the responses were only short lived, and survival was not significantly improved.

One study has suggested survival benefit from the addition of resistance modifier (Figueredo et al; 1990). In this non-randomised study, verapamil (360 or 480 mg/day) and tamoxifen (80 or 100 mg/day) were given in addition to combination chemotherapy with doxorubicin, vincristine and etoposide (DVE), to 58 patients with extensive small cell lung cancer. If patients were resistant to DVE or relapsed after responding to DVE they were treated with cyclophosphamide and cisplatin (CC) as second line therapy.

Cardiovascular toxicity was not significant, presumably related to the relatively modest doses of oral verapamil given. Verapamil plasma levels were not measured.

The initial response rate was quite high for this group of patients (complete 24%, overall 58%). Median survival was 46 weeks. This compares favourably with historic controls and does suggest that this combination of resistance modifiers may be active in conjunction with combination chemotherapy in extensive SCLC.

A group from Sydney, Australia are also conducting a randomised study of verapamil given in addition to chemotherapy in small cell lung cancer. They have reported their intermediate results (Wheeler et al., 1988) and the study remains in progress (Bell, 1990, written communication). The total doses of chemotherapy given were relatively low and although response rates were fair (56% overall response rate), median survival was disappointing (only 9 months in the limited disease patients and only 3 months in the extensive disease patients). These results may relate to sub-maximal doses of chemotherapy. It is therefore difficult to establish the significance of these intermediate results.

The only other P-glycoprotein active resistance modifier to be used to date in the clinical setting is trifluoperazine (Miller et al., 1988). In this study the authors reported 7 responses to chemotherapy with doxorubicin plus trifluoperazine in 36 patients with resistant tumour. Again it was not possible to achieve levels of modifier in the clinic that had been found to be optimally active in vitro without unacceptable side effects (extra-pyramidal toxicity). Plasma levels of trifluoperazine achieved in the clinic were only 4-130 ng/ml compared to levels optimally active in vitro (400-2000 ng/ml, Ganapathi et al., 1984; Ganapathi & Grabowski, 1988).

Thus there has been no published large-scale randomised study to assess if giving a resistance modifier in addition to chemotherapy is of clinical benefit. Such a study was therefore undertaken to see

if this approach could have an impact on the drug resistance seen in the clinic in patients with SCLC.

Although patients with resistant tumours appear to be the best population in whom to investigate the activity of resistance modifiers, this is a heterogeneous group of patients who have been heavily pretreated and who have advanced disease. Thus the trial design was inevitably a compromise between the constraints of realistic clinical practice and scientific objectives, and so the activity of verapamil in previously untreated SCLC patients was investigated. Information from the previously reported clinical pilot studies of resistance modifiers was utilised in the trial design.

A four-drug combination with documented efficacy in small cell lung cancer was used. Doxorubicin, vincristine and etoposide are all active single agents in SCLC and have been used widely in clinical studies (Hansen, 1987). These drugs are known to be involved in resistance in the MDR phenotype. Cyclophosphamide, the fourth drug used in the chemotherapy protocol is not associated with MDR (Cantwell et al., 1988). Verapamil was combined with chemotherapy from the outset, when the resistant population is expected to be small.

The major end-points of this clinical study were to assess the feasibility of giving oral verapamil in conjunction with combination cytotoxic chemotherapy in patients with SCLC, to assess any possible enhanced normal tissue toxicity and to examine for an increased response rate and/or improved survival. If effective one might not

expect the resistance modifier to change response rate because of the presumed initially low proportion of resistant cells within the tumour. On the other hand, a relatively small decrease in the resistant tumour cell population would subsequently become apparent as prolongation of time to progression and improved survival. The structure of the current study comprising 226 patients is described.

METHODS

Patient Selection

Patients with histologically proven small cell lung cancer aged 70 or less and with ECOG performance status of 0,1 or 2 were eligible. Initially the study was restricted to patients with only limited disease, but after the first 60 patients had been entered, patients with extensive disease suitable for intensive combination induction chemotherapy were also eligible. All patients had adequate bone marrow and hepatic function and had had no previous chemotherapy or radiotherapy. Eligible patients had no active cardiac disease, and had not been on beta-blocker or prior calcium antagonist therapy. All patients gave informed consent.

Registration, Stratification, Randomisation and Data Collection

Patients were registered at the clinical trials office and were stratified according to disease extent prior to randomisation. Nine Centres from the West of Scotland, one from Aberdeen and also one from Northern Ireland entered patients into this study under the auspices of the West of Scotland Lung Cancer Research Group. Following entry to the study, detailed treatment and follow-up record forms were completed and collected at the West of Scotland Clinical Trials Office at the Beatson Oncology Centre (Western Infirmary,

Glasgow) for subsequent analysis.

Pre-Treatment Studies

These included a full clinical examination, including pulse and lying and standing blood pressure. A full blood count and biochemical screen (urea, electrolytes and liver function tests) were checked along with a pre-treatment chest x-ray and bronchoscopy.

Treatment Design

Patients who were randomised to receive verapamil, were given oral verapamil 120 mg 6 hourly (i.e. 480 mg/day) for a total of five days, beginning two days prior to chemotherapy to achieve steady-state levels of verapamil at the time of cytotoxic treatment. Capsules were not counted to check compliance. However, blood samples were obtained during verapamil treatment from 18 patients. (See below).

Patients were treated with cyclophosphamide (750 mg/m^2 by i.v. bolus), doxorubicin (40 mg/m^2 by i.v. bolus), vincristine (1.4 mg/m^2 by i.v. bolus) on Day 1 and etoposide (75 mg/m^2 , as a one hour intravenous infusion) on Days 1,2 and 3. Verapamil was continued (120 mgs 6 hourly) for Days 1,2 and 3 of chemotherapy. The control patients (treated with the same intravenous chemotherapy) were not treated with placebo capsules and thus the study was not placebo controlled.

Patients received 4 courses of chemotherapy repeated at 3 weekly intervals unless there was significant toxicity necessitating withdrawal or there was evidence of disease progression. Patients who, at restaging after 4 courses were felt to have entered complete remission (see below), received consolidative radiotherapy comprising 4000 cGy in 3 weeks given to the primary site(s) of thoracic disease and 3000 cGy in 2 weeks given simultaneously to the whole brain (as prophylaxis against cerebral relapse).

Dose Modification

Chemotherapy was given at the doses listed above, if on Day 1 the white cell count (wbc) was $>3.0 \times 10^9/l$ and platelets were $>100 \times 10^9/l$. If these parameters had not been attained, treatment was postponed for 1 week and full doses given when parameters were satisfactory. Treatment was postponed for a further week if necessary, but if delay was longer than 2 weeks, protocol chemotherapy was discontinued.

If the nadir (mid-cycle) wbc, measured on Day 10, was $<1.0 \times 10^9/l$ or if nadir platelets were $<30 \times 10^9/l$, the doses of cytotoxic drug in subsequent courses of chemotherapy were reduced to cyclophosphamide 600 mg/m², doxorubicin 30 mg/m² on day 1, and etoposide 50 mg/m² on days 1,2 and 3.

Follow-up Studies and Restaging

For patients in the verapamil treatment arm, pulse and blood pressure were checked immediately prior to starting the first course of verapamil and again on Day 1 of chemotherapy, (i.e. the third day of oral verapamil treatment). In addition an ECG was checked at this time, and subsequently checked with every course of chemotherapy in patients on the verapamil arm.

Full restaging was performed after 4 cycles of chemotherapy had been completed. This comprised repeat clinical (including repeat haematological and biochemical parameters) and radiological examinations. In those patients thought to have entered complete remission, re-staging bronchoscopy was performed to confirm the absence of any residual endobronchial tumour. Response was divided into complete (CR), partial (PR) and none (NR) as defined by WHO criteria. Survival was measured as time from randomisation to time of death.

Verapamil Levels

A total of 72 blood samples were obtained from 18 patients (range 1-11 samples per patient), selected from the verapamil treatment group. Verapamil concentrations in plasma were estimated by an HPLC assay with fluorescence detection (Cole et al., 1981).

Confirmation of Response

Because the study was not blinded or placebo controlled, and because it was the individual clinicians who were responsible for assessing response at restaging, observer bias in assessment of response was possible. Therefore, a total of 64 pre-therapy and restaging x-rays from 7 participating institutions were examined by an independent radiologist. The re-staging x-rays from the pair were always examined first, and only if entirely normal was a complete radiological response accepted.

Statistical Methods

The study was stratified for disease extent. A patient was randomised by a telephone call to the West of Scotland Clinical Trials Unit. The randomisation list was constructed using random permuted blocks of length 6. Comparisons of pretreatment characteristics and survival were based on all randomised eligible patients. All other comparisons used randomised eligible patients who started protocol treatment. Categorical variables were compared mainly using Pearson's chi-square test (with no continuity correction). Categories were combined if necessary to make all expected values greater than or equal to 5. If it was not possible to combine categories to make all expected values greater than or equal to 5 then Fisher's exact test was used on the appropriate 2 x 2 table. When overall response was

compared stratification according to disease extent was included in the analysis and the p-values were calculated using the Mantel-Haenszel test. The Mann-Whitney U-test was used for the comparison of continuous variables such as age and total cumulative dose. When pulse and blood pressure measurements were compared before and after verapamil, Wilcoxon's signed rank sum test was used. Kaplan-Meier estimates were used for survival curves. Survival curves were terminated when 5 patients were at risk. Survival was measured from time of randomisation and all causes of death have been included. Comparison of survival was by the Mantel-Haenszel stratified log-rank test, with stratification based on extent of disease.

RESULTS

Patient Demography

Recruitment to this study is complete and a total of 226 patients have been entered. Of these, six patients were found to be ineligible soon after randomisation because they were found to have extensive disease at a time when the study was only open to patients with limited disease (Table 7.1). One patient aged 73 was also strictly ineligible but was treated according to protocol and has been included in the analysis.

In terms of disease stage, similar numbers of extensive and limited disease patients were randomised to each group ($p = 0.815$). An even spread of performance status at randomisation was found between the two treatment arms ($p = 0.752$). No significant difference was found between the two arms of the study with regard to the prognostic indicators described by Souhami et al. (1985) ($p = 0.827$) (Table 7.2).

Total Drug Doses and Number of Cycles of Protocol Treatment

Table 7.3 indicates that similar numbers of cycles of protocol treatment were administered in the verapamil and control arms ($p = 0.918$). Considering the total cumulative doses of cytotoxic drugs administered, the only suggestion of a difference was with

	Verapamil	Control
Randomised	113	113
Randomised + eligible	111(a)	109(b)
Randomised, eligible and started protocol treatment	107(c)	107(d)

- a. Two patients excluded because found to have extensive disease before such patients were eligible.
- b. Four patients excluded because found to have extensive disease before such patients were eligible.
- c. One patient refused treatment. One patient died before receiving any chemotherapy. One patient on beta-blockers and not given verapamil. One patient hypotensive and not given verapamil.
- d. One patient already on verapamil. One patient given verapamil in error.

Table 7.1

Details of patients entered into study.

Arm		Min	25% ile*	Median	75% ile*	Max	No. of Pats	P-value
Age	Verapamil	35	53	59	64	73	111	.495
	Control	37	55	59	65	69	109	
		Verapamil		Control		P-value		
Performance status (ECOG)	0	22.5% (25)		25.7% (28)		.752		
	1	65.8% (73)		65.1% (71)				
	2	11.7% (13)		9.2% (10)				
		100.0% (111)		100.0% (109)				
Sex	Male	55.9% (62)		59.6% (65)		.571		
	Female	44.1% (49)		40.4% (44)				
		100.0% (111)		100.0% (109)				
Disease extent	Limited	75.7% (84)		74.3% (81)		.815		
	Extensive	24.3% (27)		25.7% (28)				
		100.0% (111)		100.0% (109)				
Souhami categories	Good	28.1% (27)		29.5% (28)		.827		
	Moderate	43.8% (42)		46.3% (44)				
	Poor	28.1% (27)		24.2% (23)				
		100.0% (98)		100.0% (95)				

*25% ile and 75% ile refer to the lower and upper quartiles.

Table 7.2

Details of pre-treatment patient characteristics. Figures in parentheses are actual number of patients.

Total cumulative dose of doxorubicin*						
	Min.	25%ile**	Median	75%ile**	Max.	P-value
Verapamil	38	123	154	160	175	0.586
Control	38	119	156	160	175	
Total cumulative dose of fluorouracil*						
	Min.	25%ile	Median	75%ile	Max.	P-value
Verapamil	1.1	3.7	4.4	4.9	6.0	0.422
Control	0.9	4.0	4.4	5.0	6.9	
Total cumulative dose of cyclophosphamide*						
	Min.	25%ile	Median	75%ile	Max.	P-value
Verapamil	291	2084	2500	2893	3429	0.106
Control	571	2199	2738	2973	3222	
Total cumulative dose of etoposide*						
	Min.	25%ile	Median	75%ile	Max.	P-value
Verapamil	75	600	658	675	943	0.472
Control	73	626	660	688	900	
	Verapamil		Control		P-value	
No. of cycles of	1	8.4% (9)	8.4% (9)		0.918	
Protocol treatment	2	3.7% (4)	5.6% (6)			
	3	9.4% (10)	10.3% (11)			
	4	78.5% (84)	75.7% (81)			
		100.0% (107)	100.0% (107)			

* Total cumulative dose expressed as mg per metre squared.
 ** 25%ile and 75%ile refer to lower and upper quartiles.

Table 7.3

Total cumulative doses of cytotoxic drugs administered and number of cycles of protocol treatment given in each arm of the study.

cyclophosphamide, where the median cumulative dose in the verapamil arm was lower (2500mg cf: 2738mg; $p = 0.106$). This may have related to the higher incidence ($p = 0.031$) of dose reductions after course 1 in the verapamil treatment arm (see Toxicity, Table 7.9 below).

Reasons for Discontinuing Chemotherapy

There were a similar number of patients who stopped chemotherapy after 2 courses in both treatment arms. There were a similar number of deaths during treatment in each arm. Reasons for discontinuing chemotherapy and the causes of death during treatment in each treatment arm are listed in Table 7.4. It can be seen there were no significant differences in the number of patients who did not complete chemotherapy or the reasons for discontinuing chemotherapy.

Toxicity

The worst toxicity during any one patient's treatment was recorded. There were no statistically significant differences in general toxicities between the two arms, except for more severe alopecia in the verapamil treatment group ($p = 0.045$) (Table 7.5).

Apart from a small, but statistically significant fall in systolic and diastolic blood pressure following the first course of treatment

	Verapamil	Control
Progressive disease	7	10
Patient refusal	2	2
Excessive toxicity	3	2
Dead (drug toxicity)	5	3
Dead (non-cancer cause)	2	4
Dead (cause unknown)	2	3
Other reasons	2 *	2**

* One patient developed phobic reaction to i.v. line.
In the other case clinician decided that the tumour was chemoresistant and switched to XRT.

** One patient stopped because of general deterioration in health. The other patient developed an empyema.

Table 7.4

Reasons for discontinuing treatment before 4th cycle
of chemotherapy.

	WHO grade	Verapamil		Control		P-value
Nausea	0	9.9%	(10)	5.7%	(6)	.553
	1	29.7%	(30)	30.5%	(32)	
	2	25.7%	(26)	23.8%	(25)	
	3	33.7%	(34)	36.2%	(38)	
	4	1.0%	(1)	3.8%	(4)	
		100.0%	(101)	100.0%	(105)	
Oral	0	71.0%	(71)	77.7%	(80)	.528
	1	19.0%	(19)	15.5%	(16)	
	2*	6.0%	(6)	3.9%	(4)	
	3*	3.0%	(3)	2.9%	(3)	
	4*	1.0%	(1)	0.0%	(0)	
		100.0%	(100)	100.0%	(103)	
Haemorrhage	0	98.0%	(98)	98.1%	(101)	1.000**
	1*	1.0%	(1)	0.0%	(0)	
	2*	1.0%	(1)	1.0%	(1)	
	3*	0.0%	(0)	0.0%	(0)	
	4*	0.0%	(0)	1.0%	(1)	
		100.0%	(100)	100.0%	(103)	
Alopecia	0	7.1%	(7)	9.5%	(10)	.045
	1	1.0%	(1)	5.7%	(6)	
	2	11.1%	(11)	20.0%	(21)	
	3	80.8%	(80)	64.8%	(68)	
	4	0.0%	(0)	0.0%	(0)	
		100.0%	(99)	100.0%	(105)	
Fever	0	88.0%	(88)	93.2%	(96)	.203
	1*	9.0%	(9)	1.9%	(2)	
	2*	3.0%	(3)	4.9%	(5)	
		100.0%	(100)	100.0%	(103)	
Neurotoxicity	0	64.7%	(64)	64.1%	(66)	.978
	1	29.3%	(29)	29.1%	(30)	
	2*	5.1%	(5)	4.9%	(5)	
	3*	1.1%	(1)	1.9%	(2)	
		100.0%	(99)	100.0%	(103)	
Cardiac	0	98.0%	(98)	96.1%	(99)	.683**
	1*	0.0%	(0)	2.9%	(3)	
	2*	1.0%	(1)	0.0%	(0)	
	3*	1.0%	(1)	0.0%	(0)	
	4*	0.0%	(0)	1.0%	(1)	
		100.0%	(100)	100.0%	(103)	

* These categories combined for calculating the P-value.

** This P-value obtained from Fisher's exact test.

Table 7.5

Toxicity (based on all randomised eligible patients who received at least one course of protocol treatment). Figures in parentheses refer to the actual number of patients.

with verapamil [$p = <0.001$ (systolic BP); $p = 0.005$ (diastolic BP)] there was no evidence of increased cardiovascular toxicity in the verapamil arm (Table 7.6). One patient in the control arm died of an acute myocardial infarction. One patient in the verapamil treatment arm developed transient 1st degree A-V block during the first course of verapamil, but this did not prevent further treatment with verapamil and the heart block did not recur.

With regard to myelosuppression Table 7.7 shows that the median nadir white cell count after cycle 1 of chemotherapy was lower in the verapamil arm than in the control arm. This did not quite reach statistical significance ($p=0.065$) and overall there was no significant difference in haematological toxicity (Table 7.8). Since there was a suggestion of a difference in nadir white cell counts after course 1 between the verapamil and control arms, the incidence of dose reductions in the two treatment arms after course 1 and after subsequent courses of chemotherapy was investigated. There were significantly more dose reductions after course 1 in the verapamil treatment arm ($p = 0.031$) (Table 7.9).

Response

Response data, for all the patients in each treatment arm are shown in Table 7.10. No statistically significant differences in overall response were seen. Patients with extensive disease had a bigger gain in overall response rate in the verapamil arm (87% cf: 70% for

		Verapamil			P value
Cycle 1	Systolic BP	Before 130 (100-210)* n=83	After 120 (90-170) n=80	After-Before -10 (-100 to 26) n=75	<0.001
	Diastolic BP	75 (48-130) n=83	70 (48-100) n=80	0 (-60 to 20) n=75	0.005
	Pulse	84 (60-130) n=73	80 (58-115) n=75	-1 (-34 to 20) n=68	0.066

* Results are median values (and range)

Table 7.6

Cardiovascular parameters measured before and after 3 days treatment with verapamil.

		Verapamil	Control	P-value
After Cycle 1	Hb WBC Platelets	12.5* (9.2 - 15.5, n=83) 1.6 (0.1 - 2.8, n=84) 165 (37 - 598, n=83)	12.4 (8.7 - 15.7, n=84) 2.0 (0.3 - 2.9, n=84) 179 (35 - 544, n=82)	0.557 0.065 0.646
After Cycle 2	Hb WBC Platelets	11.7 (8.5 - 16.4, n=70) 2.2 (0.2 - 9.3, n=70) 199 (51 - 546, n=68)	11.5 (8.2 - 15.6, n=74) 2.1 (0.6 - 9.8, n=74) 218 (65 - 730, n=74)	0.640 0.539 0.371
After Cycle 3	Hb WBC Platelets	11.0 (7.5 - 16.4, n=70) 2.0 (0.3 - 8.7, n=71) 189 (40 - 541, n=69)	10.7 (7.7 - 18.3, n=74) 2.3 (0.1 - 13.5, n=74) 193 (49 - 546, n=74)	0.285 0.266 0.715
After Cycle 4	Hb WBC Platelets	10.5 (7.3 - 15.0, n=52) 2.3 (0.4 - 9.7, n=53) 198 (52 - 432, n=52)	10.2 (4.2 - 14.6, n=50) 2.3 (0.7 - 11.6, n=50) 189 (40 - 509, n=49)	0.320 0.955 0.711

* Results are median values and ranges are shown in parentheses.

n is the number of observations.

Table 7.7

Nadir blood count values measured 10 days after completion of each of the 4 cycles of chemotherapy.

	WHO Grade	Versapam [†]	Control	P-value
Haemoglobin	0	37.0% (34)	35.5% (33)	.289
	1	43.5% (40)	35.5% (33)	
	2*	15.2% (14)	23.7% (22)	
	3*	2.2% (2)	3.2% (3)	
	4*	2.2% (2)	2.2% (2)	
		100.0% (92)	100.0% (93)	
WBC	0*	5.4% (5)	5.4% (5)	.392
	1*	4.4% (4)	5.4% (5)	
	2	16.3% (15)	18.3% (17)	
	3	42.4% (39)	50.5% (47)	
	4	31.5% (29)	20.4% (19)	
		100.0% (92)	100.0% (93)	
Platelets	0	71.7% (66)	75.3% (70)	.777
	1	14.1% (13)	14.0% (13)	
	2*	8.7% (8)	6.5% (6)	
	3*	5.4% (5)	4.3% (4)	
		100.0% (92)	100.0% (93)	

* These categories combined for calculating the P-value.

Table 7.8

Worst recorded haematological toxicity, estimated from nadir blood counts after all courses of chemotherapy. Figures in parentheses refer to actual number of patients.

		Verapamil	Control	P-value
Chemotherapy Dose Reductions (after course 1)	Reduced	21.4% (21)	10.2% (10)	0.031
	Not Reduced	78.6% (77)	89.8% (88)	
		100.0% (98)	100.0% (98)	
Delays in Chemotherapy (after course 1)	Delayed	19.4% (19)	13.3% (13)	0.246
	Not Delayed	80.6% (79)	86.7% (85)	
		100.0% (98)	100.0% (98)	
Chemotherapy Dose Reductions (ever)	Reduced	29.9% (32)	19.6% (21)	0.082
	Never Reduced	70.1% (75)	80.4% (86)	
		100.0% (107)	100.0% (107)	
Delays in Chemotherapy (ever)	Delayed	31.8% (34)	28.0% (30)	0.550
	Never Delayed	68.2% (73)	72.0% (77)	
		100.0% (107)	100.0% (107)	

Table 7.9

Incidence of dose reductions and delays after course 1 of chemotherapy and during the entire treatment period. Figures in parentheses refer to actual number of patients.

		Verapamil	Control	P-value
Response*	CR	38.5% (37)	27.1% (26)	
	PR	44.8% (43)	53.1% (51)	
	No change	7.3% (7)	7.3% (7)	
	Progressive Disease	9.4% (9)	12.5% (12)	
		100.0% (96)	100.0% (96)	
	CR + PR	83.3% (80)	80.2% (77)	
Estimated difference in percentage of overall (CR+PR) responders (Verapamil - Control)		= 3.1%**		
95% c.i. for above difference		= -8.1% - 14.3%		
		Verapamil	Control	
Unevaluable	Dead before 12 weeks (non-cancer cause)	2	4	
	Dead before 12 weeks (cause unknown)	2	3	
	Dead before 12 weeks (drug toxicity)	5	3	
	Assessment not done	2	1	
	Did not start on protocol treatment	4	2	

* Expressed as percentage of evaluable patients.

** This difference is calculated allowing for the stratification on disease extent.

Table 7.10

Response data at 12 weeks as defined by WHO criteria for all patients. (CR=complete response, PR= partial response).

The actual number of patients are shown in parentheses. Unevaluable patients and reasons for this are also shown.

controls) compared to patients with limited disease (82.2%, verapamil cf: 83.3% for controls). This however was not statistically significant and could easily relate to random fluctuation.

Confirmation of Response

There was agreement between the clinicians' and the radiologist's assessment of response in 40 of 64 cases (Table 7.11). There were a total of 11 "downgrades" [5/27 in the verapamil arm (18.5%) and 6/37 in the control arm (16.2%)]. In the case of the "upgrades" i.e. an improvement in response rating after independent radiological assessment, there were more in the control arm (24.3%) than in the verapamil treatment arm (14.8%). Overall there was no statistically significant difference in the distribution of regradings between the two treatment arms ($p = 0.646$).

Survival

Survival curves for the patients, divided according to disease extent at presentation and according to treatment group (verapamil or control) are shown in Figure 7.1. As expected, patients with extensive disease show a worse survival pattern. There is no significant difference in the survival curves between the control and the verapamil treatment arms in limited disease patients. In extensive disease patients verapamil does appear to confer some survival

	Verapamil	Control
Downgrades	18.5% (5)	16.2% (6)
Same	66.7% (18)	59.5% (22)
Upgrades	14.8% (4)	24.3% (9)

Table 7.11

Re-evaluation of radiological response assessment by an independent radiologist.

Figures in parentheses refer to actual numbers of x-ray pairs examined.

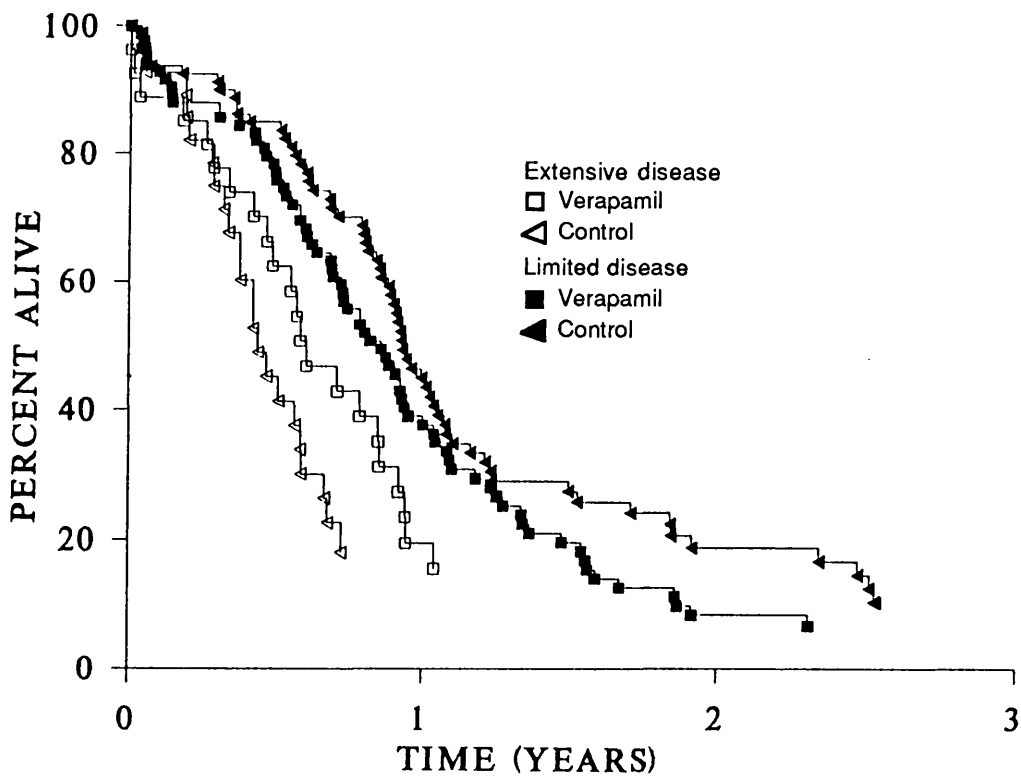


Figure 7.1

Actuarial survival curves for patients in the clinical study. Patients have been divided according to disease extent (limited or extensive) and treatment group (verapamil or control).

advantage, but this does not approach statistical significance.

The analysis of the survival curves in terms of median survival and death rate is shown in Table 7.12. This confirms that there is no significant difference in survival between the verapamil treatment and the control arms for the group as a whole ($p=0.943$). Also listed in Table 7.12 are the causes of death. There were no significant differences between the verapamil and control arms. The majority of patients have died of tumour progression.

Verapamil Levels

Median verapamil concentration for the 18 patients studied was 252 ng/ml (0.38 μ M) with wide inter-patient variation.

Arm	Disease Status	No. of Patients	No. of Deaths	Median Survival	95% c.i. for median survival
Verapamil	Limited	84	75	45 weeks	36-52 weeks
Control	Limited	81	62	48 weeks	44-56 weeks
Verapamil	Extensive	27	25	32 weeks	24-48 weeks
Control	Extensive	28	24	23 weeks	18-31 weeks
Verapamil	All	111	100	41 weeks	36-48 weeks
Control	All	109	86	44 weeks	36-49 weeks
Relative death rate (Verapamil/Control)			=	1.17	P = .290
95% c.i. for relative death rate			=	0.87 - 1.57	
Causes of Death					
		Verapamil	Control		
Drug Toxicity		6	3		
Tumour progression		86	73		
Non-cancer cause (without tumour)		0	1		
Non-cancer casue (with tumour)		3	4		
Not known		5	5		

Table 7.12

Survival data and causes of death.

DISCUSSION

This is the first large-scale randomised study to examine the feasibility and effects, in terms of toxicity, response and survival, of adding a resistance modifier to combination cytotoxic chemotherapy.

The study population was well balanced in terms of disease extent and the prognostic indices defined by Souhami (Souhami et al., 1985). Although there was not a significant effect on response and survival from the addition of verapamil to chemotherapy, the regime was well tolerated.

Toxicity

Toxicity was quite predictable and acceptable, with no evidence of increased toxicity, except for a trend to more severe alopecia and a tendency to greater myelosuppression following the first course of chemotherapy in the verapamil treatment arm. The reasons for this are uncertain. Indeed these findings may simply relate to chance differences.

As P-glycoprotein has been shown to be present in normal tissues (Fojo et al., 1987B; Thiebaut et al., 1987) this has possible implications for the use of verapamil in addition to chemotherapy. In particular there is theoretical concern that bone marrow cells might become more sensitive to the myelosuppressive effects of cytotoxic drugs. However, at least in vitro, this does not appear to be the

case. Fine et al. (1987) showed no increase in cytotoxicity with doxorubicin in normal human marrow cells in vitro. Similarly, Yalowich et al. (1985) failed to demonstrate an increase in cytotoxicity in normal bone marrow if verapamil was given in addition to doxorubicin or vincristine, although this group did demonstrate some enhanced toxicity when verapamil was combined with etoposide.

In the small number of pilot clinical studies reported (Benson III et al., 1985; Dalton et al., 1989; Miller et al., 1988; Ozols et al., 1987A; Presant et al., 1986) there was no evidence of increased marrow toxicity with modifier. However, recently Figueredo et al. (1990) have suggested that the increased myelosuppression (white cell and platelet counts not measured in all cases) and high serious infection risk they encountered in their non-randomised study may relate to the addition of resistance modifier to chemotherapy.

It has previously been reported that verapamil can increase plasma levels of doxorubicin and reduce doxorubicin clearance (Kerr et al., 1986). Thus a pharmacokinetic effect might explain the increased alopecia and lower nadir white cell counts after the first course of chemotherapy in the patients treated with verapamil. In this regard the greater incidence of dose reductions after the first course of chemotherapy in the verapamil arm patients is noteworthy. This in part relates to the lower nadir white cell counts in the verapamil treated patients, but may also be an indication of the prescribing clinicians' heightened caution in the management of those patients receiving verapamil. Although the criteria for dose reduction

were clearly specified in the protocol, and the majority of dose reductions after course 1 were confirmed to be on the basis of low Day 10 white cell counts, there may have been a tendency by the clinicians to err on the side of caution in those patients treated with verapamil.

There was no evidence of cardiovascular toxicity caused by verapamil in this study and this presumably relates to the relatively low oral doses, with consequently low plasma verapamil levels. The verapamil levels achieved in patients in this study (median 252 ng/ml) are well below those optimally active in vitro (over 3000 ng/ml). In those clinical studies where higher verapamil levels were achieved by large doses of intravenous verapamil, there was an unacceptable incidence of cardiovascular toxicity (Benson III et al., 1985; Dalton et al., 1989; Ozols et al., 1987A). There was no evidence of increased doxorubicin cardiac toxicity in the verapamil treated patients. However, the total doses of doxorubicin used in this study (maximum cumulative dose less than 320 mg) would not be expected to lead to cardiac toxicity.

Response

There were no statistically significant differences in response in the two arms of the study. However, in the extensive disease patients there was an 87% overall response rate in the verapamil treatment arm, compared with only 71% in the control arm. The

reasons for this remain unclear. These observations could quite easily relate to random fluctuation. Alternatively they may relate to the pharmacokinetic effect already discussed (Kerr et al., 1986). Indeed if verapamil was acting solely on resistant tumour sub-populations (presumably relatively small in number at presentation) one would not expect any difference in response rates between the verapamil and control arms.

Because the study was not placebo-controlled and blinded an independent radiologist reviewed in a blind fashion a selection of pre-therapy and restaging chest x-rays. The restaging x-rays were always examined first of the pair, and only if this was entirely normal did the radiologist regard the response as complete. As mentioned previously, if the patient was regarded as having had a complete response on the x-ray, a restaging bronchoscopy to confirm a CR was performed. Table 7.11 shows that the concordance rate between the clinicians' assessment of response and that of the radiologist was fair, with agreement in 40 out of 64 cases (62.5%). With regard to those patients whose radiological response was regraded following independent assessment no significant difference in the distribution of regradings between the two treatment groups was found.

Survival

There was no difference in survival noted between the treatment (verapamil) and control arms in this study. However, in

patients with extensive disease there was a difference in median survival between the verapamil arm (32 weeks) and the control arm (23 weeks). This would be a natural consequence of the higher overall response rate already noted in extensive disease patients treated with verapamil. However the difference does not approach statistical significance. Indeed the median survival of only 23 weeks in the control arm is rather disappointing, rather than the median survival in the verapamil treatment arm being strikingly good (as discussed in Figueredo et al., 1990).

Criticism of Study Design

A number of criticisms of the study design have become apparent during analysis. These concern the study population, the failure to placebo-control or blind the trial, the failure to assess response independently and the failure to consider the importance of achieving adequate levels of modifier in the clinic.

To rapidly assess clinical activity of a resistance modifier, the patients being studied should ideally have relapsed disease. However, this is a heterogeneous group of patients who have been pre-treated and who have advanced disease. Previously untreated patients felt suitable for intensive combination chemotherapy were therefore studied. Although the majority (75%) of patients had limited disease only 29% fell into Souhami's (1985) good prognosis category. Thus the median survival of only 45-48 weeks in the limited disease

patients in this study is not unexpected.

As the clinician who treated the patient and assessed response was aware of patient randomisation and as there were no dummy preparations used, the study was neither blinded or placebo-controlled. Moreover compliance with treatment in the verapamil arm was not checked by capsule counting.

In an attempt to overcome these shortcomings an independent assessment of response was undertaken in conjunction with an independent radiologist. In general, agreement was found between the clinicians' and the radiologist's assessment of response, although a tendency to underestimate response in the control arm was noted. In addition, in a group of 18 selected patients, plasma levels of verapamil were measured. Although levels were more than 10-fold below those which are known to be maximally active in SCLC *in vitro*, satisfactory verapamil levels were achieved, considering the oral dosage regime that was administered. This relatively low dose of verapamil was chosen to avoid excessive cardiovascular toxicity.

Summary

This study has shown it is possible to treat patients undergoing chemotherapy for small lung cancer with verapamil. There was no evidence of increased toxicity save for slightly more severe alopecia and a lower median nadir white cell count following the first course of chemotherapy in the verapamil treated patients. Whether these

effects relate to a pharmacokinetic phenomenon (Kerr et al., 1986) or whether they relate to heightened normal tissue toxicity because of the possible presence of P-glycoprotein in hair follicles and normal bone marrow cells or whether they simply relate to chance remains unclear.

In terms of response and survival, results in both treatment arms are similar. However, there were more dose reductions in the verapamil treated patients after the first course of chemotherapy. Thus it appears that verapamil may be having some effect despite achieving plasma levels of verapamil in the clinic that were substantially lower than levels optimally active in the SCLC cell lines in vitro. Although no clear dose-response has been demonstrated in terms of the resistance modifying activity of verapamil in the clinic, such a relationship does exist in the laboratory (Chapter 6). Thus one could speculate that with higher plasma levels of verapamil one might anticipate greater resistance modifying activity, but such an approach is limited by cardiovascular toxicity (Ozols et al., 1987A).

In summary, the addition of verapamil to cytotoxic chemotherapy in patients with small cell lung cancer is quite feasible, without undue toxicity with the dose of verapamil used. However no improvement in response or survival has been noted. At best it appears that the addition of verapamil reduces the death rate by only 13%. This may relate to failing to achieve blood levels of verapamil in the clinic that might be expected to be maximally active in terms of resistance modification. These findings, coupled with evidence of

resistance modification with verapamil in newly established small cell lung cancer cell lines in the laboratory suggest that further studies aimed at achieving higher levels of modifier to try and manipulate the MDR phenotype in the clinic merit further investigation.

CHAPTER 8

GENERAL DISCUSSION

The original aims of this thesis have, on the whole, been achieved. Nine SCLC biopsies have been established as continuous cell lines in vitro. These cell lines were derived from both untreated and from treated patients and include 2 pairs of cell lines each established from the same patient before and after a course of chemotherapy. There is a 60-fold range in the sensitivities of the cell lines to doxorubicin and for 5 of the cell lines drug sensitivity is increased by verapamil. Although the addition of verapamil to chemotherapy for patients with SCLC did not significantly improve survival, the combination was well tolerated and no adverse toxicity was observed. The results have been discussed in detail in the individual chapters and only the main points will be reviewed in this chapter.

Establishment and Characterisation of SCLC Cell Lines

A range of cell lines have been successfully established in tissue culture from biopsies of patients both prior to chemotherapy and also at time of relapse, both from the primary site and from a variety of metastatic deposits.

A great deal was learnt about the establishment of cell lines from fresh biopsy material. In particular the importance of

maintaining a high tumour cell density in the early stages became evident. Although a number of reports have described the use of enzymatic disaggregation, feeder layers, conditioned medium and serum-free medium none of these methods proved necessary for the successful establishment of a SCLC biopsy in culture.

A particularly important pair of cell lines (LS274 and LS310) have been established, representing a cell line from a pre-treatment tumour biopsy (LS274) and a cell line established from the same patient at time of relapse (LS310). This pair of cell lines may therefore be regarded as a model of drug resistance in vitro where drug resistance developed in the clinic.

These cell lines have been fully characterised and demonstrate typical features of SCLC in vitro, including characteristic appearances on pathological examination, the possession of certain marker enzyme activities, and the typical 3-p deletion seen on karyotypic analysis.

In contrast to a number of existing SCLC cell lines these lines are all at an early passage number and are thus less subject to phenotypic drift as a result of long term culture. Thus, although the clinical relevance of such tissue culture models remains unclear, these early passage newly established SCLC cell lines represent an important tool for studies of drug resistance in SCLC in vitro.

Chemosensitivity Testing

Considerable difficulties were encountered when conventional

techniques were used to measure the chemosensitivity of these cell lines. In particular, initial studies using spheroid growth measurements proved unhelpful. This related to the fact that only a few of the cell lines grew successfully in the agar-coated plates. The time required for and labour intensive nature of any one experiment was also a significant obstacle. This technique had the advantage that the intact aggregate could be studied and this was a major consideration for these cell lines since the majority could not be dispersed into a viable single cell suspension.

Subsequently a tetrazolium dye-based chemosensitivity assay was investigated as a possible means of studying drug sensitivity. A number of important deficiencies in the basic assay were identified. The major fault associated with previously published assays was the failure to demonstrate a linear relationship between cell number and MTT-formazan production. This resulted in a gross underestimation of cell numbers at high cell densities. This problem was overcome by using an increased substrate (MTT) concentration during the final incubation and by controlling the pH of the MTT-formazan product. Doxorubicin was shown to penetrate throughout the aggregates of the SCLC cell lines and MTT was reduced by the viable cell population of the aggregate. Thus the entire aggregate was exposed to the cytotoxic drug and hence MTT-formazan production could be used to measure surviving cell numbers in entire tumour aggregates. The basic assay was thus shown to be suitable for measuring chemosensitivity in the newly established SCLC cell lines.

Using this assay the chemosensitivities of the new cell lines

to doxorubicin were measured. Such studies revealed a wide (60-fold) range of sensitivity to doxorubicin. This did not appear to relate to morphology or any other characteristics of the cell lines. Neither did chemosensitivity appear to relate to the patient history from whose biopsy the cell line was established. However, considering the pair of cell lines LS274 and LS310, it does appear that in cell lines obtained from the same patient sensitivity to doxorubicin might mirror the clinical history of the patient from whose biopsies the cell lines had been established.

Subsequently the activity of verapamil in these cell lines was investigated. A 2-3 fold increase in sensitivity to doxorubicin in the presence of verapamil was found in 5 of the 9 cell lines tested. Increased sensitivity to doxorubicin in the presence of verapamil did not appear to relate to the intrinsic chemosensitivity of the cell line or the clinical history of the patient from whose biopsy the cell line had been established. Similarly increased sensitivity in the presence of verapamil did not appear to relate to cell line morphology or any other cellular characteristics. Nonetheless there was clear evidence of increased sensitivity to doxorubicin in the presence of verapamil in newly established SCLC cell lines. This finding has not been previously reported.

The mechanism of action of verapamil in the SCLC cell lines remains unclear. More recent work with these SCLC cell lines has failed to demonstrate the presence of P-glycoprotein either by Western blot or by immunocytochemistry. Previously, Cole et al. (1989) reported that verapamil can increase the sensitivity to

doxorubicin of an MDR variant of the SCLC cell line NCI-H69 which does not overexpress P-glycoprotein. Thus it appears that verapamil can modulate drug sensitivity via a mechanism that does not involve P-glycoprotein. Although this mechanism has not yet been identified it is known that verapamil can alter the energy metabolism of drug resistant cells (Broxterman et al., 1989) and of non-MDR cell lines (Plumb et al., 1989). An alteration of the ATP pool in the cell could influence the cytotoxicity of compounds such as doxorubicin. This clearly requires further detailed investigation. To date using a resistant human SCLC tumour model it has been shown that the resistance modifying activity of verapamil is dose-dependent. The concentration of verapamil that is optimally active in vitro appears to be about 6 μM . This is in contrast to the levels of verapamil which are achievable in the clinic without excessive cardiovascular toxicity (up to 1 μM). These results have implications for the application of verapamil as a resistance modifier in the clinic.

Clinical Study of Verapamil Plus Chemotherapy in SCLC

This, the only large-scale randomised clinical study of verapamil given in addition to combination chemotherapy in patients with SCLC, does not suggest an improvement in survival in the verapamil treatment arm. However, there are indications that verapamil has some activity in the clinical context. In particular following the first course of chemotherapy the median nadir white cell count was lower in the verapamil treatment arm. This resulted

in a significantly increased number of dose reductions after the first cycle of chemotherapy in the verapamil arm, compared with control. Despite this, response rates and survival were similar in both arms of the study.

These effects of verapamil in the clinic have been produced with plasma levels of modifier substantially lower than those optimally active in SCLC in vitro (0.3 uM cf: 6.6 uM). In this regard it is worth considering that verapamil may have activities in the clinic, other than those which are apparent in vitro. For example, verapamil may alter tumour blood flow (Kaelin et al., 1982) or may alter the handling and pharmacokinetics of the doxorubicin used to treat these patients (Kerr et al., 1986). Thus, although the levels obtained in the clinic were sub-optimal, some clinical activity was seen.

The addition of verapamil to combination chemotherapy in patients with SCLC is entirely feasible in terms of patient acceptability and without undue increased toxicity. Therefore, it seems reasonable to suggest that further studies of resistance modifiers should be pursued. These studies should aim to achieve levels of modifier in the clinic, similar to those levels known to be active in the laboratory. Indeed recently Chabner & Fojo (1989) have stated that clinical trials aimed at reversing multidrug resistance should be pursued as a high priority. Agents of interest include the less cardiotoxic D-isomer of verapamil and modulators such as quinidine and bepridil where clinically achievable levels are comparable to those active in the laboratory.

Possible Mechanisms of Resistance in SCLC : P-Glycoprotein Reviewed

The function of P-glycoprotein in normal cells and its regulation by substrates or inhibitors is as yet unknown. The question remains as to how important or relevant P-glycoprotein activity is to the drug resistance seen in SCLC in the clinic.

Recently Lai et al. (1989) published their work which examined MDR-1 gene expression in lung cancer (24 fresh biopsies, 67 cell lines and 10 non-tumourous lung samples). In 58% of lung tumour biopsies, including 3 of 6 SCLC biopsies significant although relatively low levels of MDR-1 gene expression were found, as was the case in 7 of 10 non-tumourous lung samples. In the lung cancer cell lines, 33% demonstrated significant, although again relatively low levels of MDR-1 gene expression. Only 4 of the 27 SCLC cell lines exhibited MDR-1 gene expression. The authors did not find an association between MDR-1 gene expression and chemosensitivity in vitro, measured using a tetrazolium dye-based assay. They concluded that the clinical multidrug resistance of many lung cancers could not be explained exclusively on the basis of expression of the MDR-1 gene. Thus it seems likely that in SCLC, recurrence of chemoresistant tumour does not relate solely to the repopulation/proliferation of cells expressing higher levels of P-glycoprotein. Nonetheless, Shen et al. (1986B) have pointed out that increased levels of MDR-1 gene expression may precede gene amplification in the development of drug resistance, and so the finding of low but detectable levels of

MDR-1 mRNA in these clinical specimens may be relevant. Certainly this study does not exclude the possibility of heterogeneity of expression of MDR-1 mRNA in individual tumour cells.

A recent clinical study published by Cantwell and colleagues (1988) has also suggested that P-glycoprotein and the MDR phenotype may not be the major cause of clinical resistance in SCLC. In this study 88 patients with SCLC were treated with the MDR drugs vincristine, doxorubicin and etoposide for 3 cycles. Those patients only showing a partial response or no response to this treatment were then treated with the non-MDR drug ifosfamide (5 gm/m²). Only 6 of the 14 patients (43%) treated with Ifosfamide showed any response to this non cross-resistant regimen and none of the responses were complete. A greater response to Ifosfamide might have been expected if MDR was the basis of the resistance, given that resistance to Ifosfamide through the MDR phenotype has not been demonstrated to date (Cantwell et al., 1988).

The recent laboratory-based report by Lai et al. (1989) and this clinical study (Cantwell et al., 1988) both suggest that over-expression of P-glycoprotein is unlikely to be the main cause of resistance in SCLC. Their results would support the findings in our clinical study which failed to demonstrate any significant benefit from the addition of verapamil to chemotherapy in the treatment of patients with SCLC, although the low levels of verapamil achieved in the clinic may also have been contributory.

Thus the role of MDR-1 gene expression and P-glycoprotein in clinical resistance in SCLC needs to be more clearly defined.

Several lines of evidence suggest that other mechanisms of resistance may be involved in the resistance of lung cancer seen in the clinic.

Alternative Mechanisms of Resistance

A number of enzyme changes, including alterations in glutathione transferases and certain topoisomerases, have been implicated as alternative mechanisms of drug resistance. In addition other enzyme alterations (Bosmann & Kessel, 1970; Bosmann, 1971) as well as altered membrane fluidity and other membrane changes (Holleran et al., 1986; Ramu et al., 1983; Wheeler et al., 1982), altered sub-cellular drug distribution (Klohs & Steinkampf, 1988; Sehested et al., 1987A,B), and ionic changes [reduced sodium pump activity (Bose & Lam, 1988) and altered calcium levels (Tsuruo et al., 1984)] have also all been implicated in drug resistance. Finally reduced drug influx has also been suggested as an alternative mechanism of resistance (Skovsgaard, 1978A,B). Most of this work was based on laboratory models of drug resistance and has been reviewed in detail by Riordan & Ling (1985).

Glutathione and Glutathione-S-Transferases

Glutathione-S-transferase (GST) comprises at least 12 isoenzymes. This phase II enzyme has long been known to bind and detoxify cellular poisons (Jakoby, 1978) and comprises a family of

dimeric proteins which conjugate electrophilic substrates with the tripeptide glutathione (GSH), thus making a less toxic and more readily excreted metabolite. The anionic isoenzyme of GST, a form found in placenta and usually present only in low levels in liver is over-expressed both in rat hyperplastic nodules (Cowan et al., 1986; Rushmore et al., 1987; Satoh et al., 1985) and in doxorubicin-resistant human breast (MCF-7) cancer cells (Batist et al., 1986; Cowan et al., 1986). A number of cytotoxic agents, including melphalan, have been shown to act as substrates for GST, and the rate of cytotoxic detoxification is increased significantly in the presence of GST catalysis (Dulik et al., 1986). Thus increased GST activity appears to be a component of resistance in some cellular systems.

Recently Moscow's and Cowan's laboratory at the National Cancer Institute, Bethesda has cloned the cDNA encoding placental GST (GSTpi). Using this cDNA probe they found a marked heterogeneity in GSTpi expression. Interestingly, as well as in placenta, this enzyme is also normally expressed in lung tissue (Moscow & Cowan, 1988). Increased amounts of GSTpi and GSTpi mRNA have been found in colon cancers compared with "normal" tissue at the resection margin (Kodate et al., 1986). Since colon carcinoma exhibits intrinsic resistance it may be that GSTpi is a marker for such resistance. Other classes of GST isoenzymes may also play a role in resistance to antineoplastic agents. For example, two different isoenzymes in the basic class of GST have been implicated in resistance to mechlorethamine (Buller et al., 1987) and alkylating agents (Manoharan et al., 1987). Furthermore

levels of glutathione, the substrate for all GSTs have been shown to be elevated in cells resistant to melphalan (Green et al., 1984A; Suzukake et al., 1982, 1983). Little is known about GST expression in SCLC. Carmichael et al. (1988B) demonstrated GST activity in a panel of lung tumours and reported that the levels of glutathione and GST were higher in NSCLC, an inherently resistant tumour type, than in SCLC cell lines.

Thus the role of GSTpi in clinical drug resistance remains unclear. In terms of doxorubicin resistance, some isoenzymes of GST possess peroxidase activity, increased levels of which could protect the tumour cell from doxorubicin toxicity, assuming that free radical generation is an important mechanism of action of doxorubicin toxicity. The peroxidase could "mop up" free radicals (Sinha et al., 1987). Thus GSTs having peroxidase activity could reduce various potential toxins, including free radicals.

It is possible to modulate GST activity both in vitro and in vivo. Buthionine sulfoximine (BSO), a synthetic amino acid analogue which inhibits the first enzyme in the GSH biosynthesis pathway, depletes intracellular glutathione which is the substrate for GST isoenzymes and glutathione peroxidase (Meister, 1983). Treatment with BSO has been shown to restore sensitivity in doxorubicin-resistant tumour cells, perhaps by permitting generation of toxic free radicals in cells which had previously been protected by glutathione (Hamilton et al., 1985). Similarly several studies of different resistant tumour cell lines have shown reversal of drug resistance after treatment with BSO, corresponding with decreases in intracellular GSH concentrations (Kramer et al., 1987;

Ozols et al., 1987B; Russo & Mitchell, 1985).

Of particular interest is recent work from Tew's group which has shown that total GST activity is elevated 4-fold in resistant compared with sensitive tumour cells (Tew & Clapper, 1988). Furthermore, using non-cytotoxic concentrations of ethacrynic acid in combination with chlorambucil, a substantial increase in the sensitivity of both sensitive and resistant rat breast carcinoma cells and human colon cancer cells was produced (Tew et al., 1988). Ethacrynic acid acts as a substrate for GST, and is known to form a glutathione conjugate through a catalytic reaction with GST (Ahokas et al., 1984). Moreover, Tew et al. (1988) demonstrated evidence of a dose-dependent decrease in GST activity with ethacrynic acid.

Topoisomerases

Certain topoisomerase enzymes have been identified as important targets for cytotoxic drugs, especially anthracyclines and podophyllotoxins. Topoisomerase II, an ATP dependent enzyme attaches to DNA and produces a double-stranded break which allows unknotting and repair of the DNA. Anthracyclines and podophyllotoxins form stable complexes with topoisomerase II and prevent it repairing DNA. In some cell lines, particularly those exhibiting rather atypical patterns of MDR, altered topoisomerase II activity has been mechanistically linked to resistance (Pommier et al., 1986).

An etoposide resistant CHO cell line that showed an atypical

MDR pattern of cross-resistance to the structurally dissimilar agents m-AMSA, mitoxantrone and doxorubicin demonstrated altered topoisomerase II activity (Glisson et al., 1986). In addition altered topoisomerase II activity has been seen in a CHO cell line selected for resistance to ellipticine which was cross-resistant to m-AMSA and etoposide (Pommier et al., 1986). Multidrug resistant cells with altered levels of topoisomerase II continue to be isolated (Deffie et al., 1989; Ferguson et al., 1988). Indeed it may be that MDR cells with increased P-glycoprotein levels may also have a component of resistance mediated via alterations in topoisomerase II.

Topoisomerases may represent the final common pathway of cytotoxicity of several different classes of antineoplastic agents. However, no study has yet determined whether altered topoisomerase activity plays a role in drug resistance in the clinical setting.

Thus the mechanisms relevant to clinical resistance in SCLC have yet to be defined. Certainly once the relevant mechanisms have been elucidated, exciting opportunities for modulation in the laboratory and the clinic will become possible.

Future Direction of Clinical Research

The ultimate goal of this laboratory research, at least from a clinician's point of view, is to enable changes in therapy that will improve median and long term survival in patients with SCLC.

Various opportunities exist for the application of what is already known about drug resistance in SCLC to be extended to the clinical setting.

In particular, if increased MDR-1 gene expression and P-glycoprotein levels are an important component, then refinement of current modifier studies with verapamil, perhaps making use of the fact that verapamil is a racemic mixture of two stereoisomers, or perhaps using alternative modifiers, would seem appropriate. If on the other hand alterations in cellular glutathione play a major component, then clinical studies aimed at interfering with GST enzyme activity are indicated.

P-glycoprotein Blockers

The main obstacle is achieving serum verapamil levels in the clinic similar to those shown to be optimally active in vitro (greater than 6 μ M). Given that a dose-response effect for verapamil in terms of resistance modifying activity with doxorubicin in SCLC in vitro has been demonstrated (Chapter 6) it seems reasonable to speculate that by increasing serum verapamil levels it might be possible to increase the activity of verapamil in SCLC in the clinic. However such dose increases with verapamil are limited by cardiovascular toxicity. A possible solution to this problem lies in the fact that verapamil is a racemic mixture of the D- and L-stereoisomers. The L-isomer is about 10-fold more active in terms of effects on cardiac conduction than the D-isomer (Curtis & Walker, 1986; Echizen et al., 1985; Triggle & Swamy, 1983).

Using a multi-drug resistant SCLC tumour model it has been shown that the resistance modifying activity of the individual stereoisomers is similar. Thus the use of D-verapamil alone might allow higher serum levels to be achieved in SCLC patients without undue cardiotoxicity. Clinical studies of D-verapamil in addition to cytotoxic chemotherapy in cancer patients are now underway.

Another possible approach to the problem of achieving levels of modifier in the clinic that are known to be optimally active in vitro might be to use an alternative modifier, such as quinidine or bepridil. Using a resistant tumour model in the laboratory it has been shown that quinidine and bepridil are capable of reversing drug resistance at concentrations that can be attained in the clinic without undue toxicity. With an oral dose of quinidine of 1gm. B.D., plasma levels of quinidine of 2-4 ug/ml are achievable, and this is the concentration that is optimally active in vitro. Clinical studies using quinidine in addition to an anthracycline in breast cancer patients are currently underway.

A final, exciting possibility for therapeutic intervention remains. This theory suggests that, in tumour cells where resistance is indeed due to P-glycoprotein, it may be possible to target monoclonal antibodies, conjugated with toxins, against the P-glycoprotein of resistant cells (Fitzgerald et al., 1987) and so use the actual mechanism that causes resistance, to overcome that resistance.

GST Antagonists

Two agents may be of clinical use in terms of overcoming GST mediated resistance, namely BSO and ethacrynic acid. Phase I clinical trials of BSO, given with the antineoplastic agent, L-phenylalanine mustard, are now underway at the Fox Chase Cancer Center in the United States (O'Dwyer, 1989, written communication). In addition at least two centres (The Fox Chase Cancer Centre and the University of California, Irvine) in the United States have started phase I studies using ethacrynic acid in combination with antineoplastic drugs including thioTEPA (Nagourney, 1989, written communication; O'Dwyer, 1989, written communication).

Concluding Comment

The difficulties of prevention and early detection mean that lung cancer will continue to be a significant clinical problem in the coming decades. Relapse with resistant tumour in small cell lung cancer remains the major obstacle in improving long-term survival in patients with this disease. In this context, it is important that studies such as those described in this thesis are extended in the pursuit of the tantalizing goal of overcoming drug resistance in small cell lung cancer.

REFERENCES

Ahokas, J.T., Davies, C., Ravenscroft, P.J. & Emmerson, B.T. (1984) Inhibition of soluble glutathione S-transferase by diuretic drugs. Biochemical Pharmacology, **33**, 1929-1932.

Aisner, J., Alberto, P., Bitran, J., Comis, R., Daniels, J., Hansen, H., Ikegami, H. & Smyth, J. (1983) Role of chemotherapy in small cell lung cancer: A concensus report of the International Association for the Study of Lung Cancer Workshop. Cancer Treatment Reports, **67**, 37-43.

Akiyama, S., Gottesman, M.M. Hanover, J.A., Fitzgerald, D.J., Willingham, M.C. & Pastan, I. (1984) Verapamil enhances the toxicity of conjugates of epidermal growth factor with *Pseudomonas* exotoxin and antitransferrin receptor with *Pseudomonas* exotoxin. Journal of Cellular Physiology, **120**, 271-279.

Akiyama, S., Fojo, A., Hanover, J.A., Pastan, I. & Gottesman, M.M. (1985A) Isolation and genetic characterization of human KB cell lines resistant to multiple drugs. Somatic Cell and Molecular Genetics, **11**, 117-126.

Akiyama, S., Tomita, K. & Kuwano, M. (1985B) The effect of calcium antagonists on the proteolytic degradation of low-density lipoprotein in HeLa cells. Experimental Cell Research, **158**, 192-204.

Akiyama, S., Shiraishi, N., Kuratomi, Y., Nakagawa, M. & Kuwano, M. (1986) Circumvention of multiple-drug resistance in human cancer cells by thioridazine, trifluoperazine and chlorpromazine. Journal of the National Cancer Institute, **76**, 839-844.

Alexander, P. (1982) 2nd Gordon Hamilton Fairley Lecture. Need for new approaches to the treatment of patients in clinical remission with special reference to acute myeloid leukaemia. British Journal of Cancer, **46**, 151-159.

Allan, S.G., Hay, F.G., McIntyre, M.A. & Leonard, R.C. (1987) Prognosis in small cell carcinoma of the lung - relationship to human milk fat globule 2 (HMFG2) antigen and other small cell associated antigens. British Journal of Cancer, **56**, 485-488.

Alley, M.C., Uhl, C.B. & Lieber, M.M. (1982) Improved detection of drug cytotoxicity in the soft agar colony formation assay through use of a metabolizable tetrazolium salt. Life Sciences, **31**, 3071-3078.

Alley, M.C. & Lieber, M.M. (1984) Improved optical detection of colony enlargement and drug cytotoxicity in primary soft agar cultures of human solid tumour cells. British Journal of Cancer, **49**, 225-233.

Alley, M.C., Scudiero, D.A., Monks, A., Hursey, M.L., Czerwinski, M.J., Fine, D.L., Abbott, B.J., Mayo, J.G., Shoemaker, R.H. & Boyd, M.R. (1988) Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. Cancer Research, **48**, 589-601.

Auerbach, O., Frasca, J.M., Parks, V.R. & Carter, H.W. (1982) A comparison of World Health Organisation (WHO) Classification of Lung Tumours by Light and Electron Microscopy. Cancer, **50**, 2079-2088.

Baillie-Johnson, H., Twentyman, P.R., Fox, N.E., Walls, G.A., Workman, P., Watson, J.V., Johnson, N., Reeve, J.G. & Bleehen, N.M. (1985) Establishment and characterisation of cell lines from patients with lung cancer (predominantly small cell carcinoma). British Journal of Cancer, **52**, 495-504.

Ballou, R.J. & Tseng, M.T. (1986) In vitro and in vivo responses of a murine transitional cell carcinoma to doxorubicin, mitoxantrone and aclacinomycin-A. Urological Research, **14**, 217-222.

Barnes, D. & Sato, G. (1980) Methods for growth of cultured cells in serum-free medium. Analytical Biochemistry, **102**, 255-270.

Barranco, S.C., Ho, D.H., Drewinko, B., Romsdahl, M.M. & Humphrey, R. (1972) Differential sensitivities of human melanoma cells grown in vitro to arabinosylcytosine. Cancer Research, **32**, 2733-2736.

Barranco, S.C., Haenelt, B.R. & Gee, E.L. (1978) Differential sensitivities of five rat hepatoma cell lines to anticancer drugs. Cancer Research, **38**, 656-660.

Barranco, S.C. & Townsend, C.M., Jr. (1986) Loss in cell killing effectiveness of anticancer drugs in human gastric cancer clones due to recovery from potentially lethal damage in-vitro. Cancer Research, **46**, 623-628.

Batist, G., Tulpule, A., Sinha, B.K., Katki, A.G., Myers, C.E. & Cowan, K.H. (1986) Overexpression of a novel anionic glutathione transferase in multi-drug resistant human breast cancer cells. Journal of Biological Chemistry, **261**, 15544-15549.

Baylin, S.B., Abeloff, M.D., Goodwin, G., Carney, D.N. & Gazdar, A.F. (1980) Activities of L-dopa decarboxylase and diamine oxidase (histaminase) in human lung cancers and decarboxylase as a marker for small (oat) cell cancer in cell culture. Cancer Research, **40**, 1990-1994.

Baylin, S.B., Gazdar, A.F., Minna, J.D., Bernal, S.D. & Shaper, J.H. (1982) A unique cell-surface protein phenotype distinguishes human small-cell from non-small cell lung cancer. Proceedings of the National Academy of Sciences of the United States of America, **79**, 4650-4654.

Bech-Hansen, N.T., Till, J.E. & Ling, V. (1976) Pleiotropic phenotype of colchicine-resistant CHO cells: cross resistance and collateral sensitivity. Journal of Cellular Physiology, **88**, 23-31.

Bech-Hansen, N.T., Sarangi, F., Sutherland, D.J. & Ling, V. (1977) Rapid assays for evaluating the drug sensitivity of tumor cells. Journal of the National Cancer Institute, **59**, 21-27.

Beck, W.T., Mueller, T.J. & Tanzer, L.R. (1979) Altered surface membrane glycoproteins in Vinca alkaloid-resistant human leukemic lymphoblasts. Cancer Research, **39**, 2070-2076.

Beck, W.T., Cirtain, M.C., Look, A.T. & Ashmun, R.A. (1986) Reversal of Vinca alkaloid resistance but not multiple drug resistance in human leukemic cells by verapamil. Cancer Research, **46**, 778-784.

Beck, W.T. (1987) The cell biology of multiple drug resistance. Biochemical Pharmacology, **36**, 2879-2887.

Beijnen, J.H., Van der Nat, J.M., Labadie, R.P. & Underberg, W.J. (1986) Decomposition of mitomycin and anthracycline cytostatics in cell culture media. Anticancer Research, **6**, 39-44.

Bell, D.R., Gerlach, J.H., Kartner, N., Buick, R.N. & Ling, V. (1985) Detection of P-glycoprotein in ovarian cancer: a molecular marker associated with multidrug resistance. Journal of Clinical Oncology, **3**, 311-315.

Bell, D. (1990) Written communication.

Benson, A.B., III, Trump, D.L., Koeller, J.M., Egorin, M.I., Olman, E.A., Witte, R.S., Davis, T.E. & Tormey, D.C. (1985) Phase I study of vinblastine and verapamil given by concurrent iv infusion. Cancer Treatment Reports, **69**, 795-799.

Bepler, G., Jaques, G., Havemann, K., Koehler, A., Johnson, B.E. & Gazdar, A.F. (1987A) Characterization of two cell lines with distinct phenotypes established from a patient with small cell lung cancer. Cancer Research, **47**, 1883-1891.

Bepler, G., Jaques, G., Koehler, A., Gropp, C. & Havemann K. (1987B) Markers and characteristics of human SCLC cell lines. Neuroendocrine markers, classical tumor markers, and chromosomal characteristics of permanent human small cell lung cancer cell lines. Journal of Cancer Research and Clinical Oncology, **113**, 253-259.

Bepler, G., Jaques, G., Neumann, K., Aumuller, G., Gropp, C. & Havemann, K. (1987C) Establishment, growth properties and morphological characteristics of permanent human small cell lung cancer cell lines. Journal of Cancer Research and Clinical Oncology, **113**, 31-40.

Berendsen, H.H., de Leij, L., de Vries, E.G., Mesander, G., Mulder, N.H., de Jong, B., Buys, C.H., Postmus, P.E., Poppema, S., Sluiter, H.J. & The, H.T. (1988) Characterization of three small cell lung cancer cells lines established from one patient during longitudinal follow-up. Cancer Research, **48**, 6891-6899.

Berger, C.L., Goodwin, G., Mendelsohn, G., Eggleston, J.C., Abeloff, M.D., Aisner, S. & Baylin, S.B. (1981) Endocrine-related biochemistry in the spectrum of human lung carcinoma. Journal of Clinical Endocrinology and Metabolism, **53**, 422-429.

Bergh, J., Larsson, E., Zech, L. & Nilsson, K. (1982) Establishment and characterization of two neoplastic cell lines (U-1285 and U-1568) derived from small cell carcinoma of the lung. Acta Pathologica Microbiologica et Immunologica Scandinavica Section A Pathology, **90**, 149-158.

Bergh, J., Nilsson, K., Dahl, D., Andersson, L., Virtanen, I. & Lehto, V.P. (1984) Expression of intermediate filaments in established human lung cancer cell lines. An indicator of differentiation and derivation. Laboratory Investigation, **51**, 307-316.

Bergh, J., Nilsson, K., Ekman, R. & Giovanella, B. (1985). Establishment and characterization of cell lines from human small cell and large cell carcinomas of the lung. Acta Pathologica Microbiologica et Immunologica Scandinavica Section A Pathology, **93**, 133-147.

Bernal, S.D., Baylin, S.B., Shaper, J.H, Gazdar, A. & Chen, L.B. (1983) Cytoskeleton-associated proteins of human lung cancer cells. Cancer Research, **43**, 1798-808.

Berry, R.J., Laing, A.H. & Wells, J. (1975) Fresh explant culture of human tumours in vitro and the assessment of sensitivity to cytotoxic chemotherapy. British Journal of Cancer, **31**, 218-227.

Bertoncello, I., Bradley, T.R., Campbell, J.J., Day, A.J., McDonald, I.A., McLeish, G.R., Quinn, M.A., Rome, R. & Hodgson, G.S. (1982) Limitations of the clonal agar assay for the assessment of primary human ovarian tumour biopsies. British Journal of Cancer, **45**, 803-811.

Bhuyan, B.K., Loughman, B.E. & Fraser, T.J. (1976) Comparison of different methods of determining cell viability after exposure to cytotoxic compounds. Experimental Cell Research, **97**, 275-280.

Biedler, J.L. & Riehm, H. (1970) Cellular resistance to actinomycin D in Chinese hamster cells in vitro: cross-resistance, radioautographic and cytogenetic studies. Cancer Research, **30**, 1174-1184.

Bird, M.C., Bosanquet, A.G., Forskitt, S. & Gilby, E.D. (1986) Semi-micro adaption of a 4-day differential staining cytotoxicity of (Disc) assay for determining the in vitro chemosensitivity of haematological malignancies. Leukaemia Research, **10**, 445-449.

Black, M.M. & Speer, F.D. (1953) Effects of cancer chemotherapeutic agents on dehydrogenase activity of human cancer tissue in vitro. American Journal of Clinical Pathology, **23**, 218-227.

Black, M.M., Zweifach, B.W. & Speer, F.D. (1953) Tetrazolium salts. A new tool in general and experimental pathology. American Journal of Clinical Pathology, **23**, 332-339.

Bogden, A.E. & Van Hoff, D.D. (1984) Comparison of the human tumor cloning and subrenal capsule assays. Cancer Research, **44**, 1087-1090.

Bogden, A.E., Griffin, W., Reich, S.D., Costanza, M.E. & Cobb, W.R. (1984) Predictive testing with the subrenal capsule assay. Cancer Treatment Reviews, **11**, Suppl. A., 113-124.

Bose, R. & Lam, H.Y. (1988) Membrane transport changes in an adriamycin-resistant murine leukemia cell line and in its sensitive parental cell line. Cancer Chemotherapy and Pharmacology, **21**, 14-18.

Bosmann, H.B. & Kessel, D. (1970) Altered glycosidase levels in drug-resistant mouse leukemias. Molecular Pharmacology, **6**, 345-349.

Bosmann, H.B. (1971) Mechanism of cellular drug resistance. Nature, **233**, 566-569.

Bottenstein, J., Hayashi, I., Hutchings, S., Masui, H., Mather, J., McClure, D.B., Ohasa, S., Rizzino, A., Sato, G., Serrero, G., Wolfe, R. & Wu, R. (1979) The growth of cells in serum-free hormone-supplemented media. Methods in Enzymology, **49**, 94-109.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, **72**, 248-254.

Broers, J.L., Carney, D.N., de Ley, L., Vooijs, G.P. & Ramaekers, F.C. (1985) Differential expression of intermediate filament proteins distinguishes classic from variant small-cell lung cancer cell lines. Proceedings of the National Academy of Sciences of the United States of America, **82**, 4409-4413.

Broers, J.L., Carney, D.N., Klein Rot, M., Schaart, G., Lane, E.B., Vooijs, G.P. & Ramaekers, F.C. (1986) Intermediate filament proteins in classic and variant types of small cell lung carcinoma cell lines: a biochemical and immunochemical analysis using a panel of monoclonal and polyclonal antibodies. Journal of Cell Science, **83**, 37-60.

Broxterman, H.J., Pinedo, H.M., Kuiper, C.M., Schuurhuis, G.J. & Lankelma, J. (1989) Glycolysis in P-glycoprotein-overexpressing human tumor cell lines. Effects of resistance modifying agents. Federation of the European Biochemical Society Letters, **247**, 405-410.

Buller, A.L., Clapper, M.L. & Tew, K.D. (1987) Glutathione-S transferases in nitrogen mustard-resistant and -sensitive cell lines. Molecular Pharmacology, **31**, 575-578.

Burchell, J., Durbin, H. & Taylor-Papadimitriou, J. (1983) Complexity of expression of antigenic determinants recognized by monoclonal antibodies HMFG-1 and HMFG-2 in normal and malignant human mammary epithelial cells. Journal of Immunology, **131**, 508-513.

Burchell, J., Wang, D. & Taylor-Papadimitriou, J. (1984) Detection of the tumour-associated antigens recognized by the mono-clonal antibodies HMFG-1 and 2 in serum from patients with breast cancer. International Journal of Cancer, **34**, 763-768.

Buys, C.H., van der Veen, A.Y. & Leij, L. de. (1983) Chromosome analysis of three cell lines established from small cell carcinoma of the lung. In Chromosomes Today ed. Bennett, M.D., Gropp, A. & Wolf, U. Vol. 8, pp 301. London: George Allen & Unwin.

Calabresi, P., Dexter, D.L. & Heppner, G.H. (1979) Clinical and pharmacological implications of cancer cell differentiation and heterogeneity. Biochemical Pharmacology, **28**, 1933-1934.

Cantwell, B.M., Bozzino, J.M., Corris, P. & Harris, A.L. (1988) The multidrug resistant phenotype in clinical practice; evaluation of cross resistance to ifosfamide and mesna after VP16-213, doxorubicin and vincristine (VPAV) for small cell lung cancer. European Journal of Cancer and Clinical Oncology, **24**, 123-129.

Carmichael, J., DeGraff, W.G., Gazdar, A.F., Minna, J.D. & Mitchell, J.B. (1987) Evaluation of a tetrazolium-based semi-automated colorimetric assay: assessment of chemosensitivity testing. Cancer Research, **47**, 936-942.

Carmichael, J., Mitchell, J.B., DeGraff, W.G., Gamson, J., Gazdar, A.F., Johnson, B.E., Glatstein, E. & Minna, J.D. (1988A) Chemosensitivity testing of human lung cancer cell lines using the MTT assay. British Journal of Cancer, **57**, 540-547.

Carmichael, J., Mitchell, J.B., Friedman, N., Gazdar, A.F. & Russo, A. (1988B) Glutathione and related enzyme activity in human lung cancer cell lines. British Journal of Cancer, **58**, 437-440.

Carney, D.N., Gazdar, A.F. & Minna, J.D. (1980) Positive correlation between histological tumor involvement and generation of tumor cell colonies in agarose in specimens taken directly from patients with small-cell carcinoma of the lung. Cancer Research, **40**, 1820-1823.

Carney, D.N., Bunn, P.A. Jr., Gazdar, A.F., Pagan, J.A. & Minna, J.D. (1981) Selective growth in serum-free hormone-supplemented medium of tumor cells obtained by biopsy from patients with small cell carcinoma of the lung. Proceedings of the National Academy of Sciences of the United States, **78**, 3185-3189.

Carney, D.N., Mitchell, J.B. & Kinsella, T.J. (1983) In vitro radiation and chemotherapy sensitivity of established cell lines of human small cell lung cancer and its large cell morphological variants. Cancer Research, **43**, 2806-2811.

Carney, D.N., Gazdar, A.F., Bepler, G., Guccion, J.G., Marangos, P.J., Moody, T.W., Zweig, M.H. & Minna, J.D. (1985A) Establishment and identification of small cell lung cancer cell lines having classic and variant features. Cancer Research, **45**, 2913-2923.

Carney, D.N., Gazdar, A.F., Nau, M. & Minna, J.D. (1985B) Biological heterogeneity of small cell lung cancer. Seminars in Oncology, **12**, 289-303.

Carter, S.K. (1984) Some thoughts on resistance to cancer chemotherapy. Cancer Treatment Reviews, **11** (Suppl. A), 3-7.

Chabner, B.A. & Fojo, A. (1989) Multidrug resistance: P-glycoprotein and its allies - the elusive foes. Journal of the National Cancer Institute, **81**, 910-13.

Chang, B.K. & Gregory, J.A. (1985) Comparison of the cellular pharmacology of doxorubicin in resistant and sensitive models of pancreatic cancer. Cancer Chemotherapy and Pharmacology, **14**, 132-134.

Chauffert, B., Rey, D., Coudert, B., Dumas, M. & Martin, F. (1987) Amiodarone is more efficient than verapamil in reversing resistance to anthracyclines in tumour cells. British Journal of Cancer, **56**, 119-122.

Chen, C.J., Chin, J.E., Ueda, K., Clark, D.P., Pastan, I., Gottesman, M.M. & Roninson, I.B. (1986) Internal duplication and homology with bacterial transport proteins in the *mdr* I (P-glycoprotein) gene from multidrug-resistant human cells. Cell, **47**, 381-389.

Cohen, M.H. & Matthews, M.J. (1978) Small cell bronchogenic carcinoma: A distinct clinicopathologic entity. Seminars in Oncology, **5**, 234-243.

Cole, S.C., Flanagan, R.J., Johnston, A. & Holt, D.W. (1981) Rapid high performance liquid chromatographic method for the measurement of verapamil and norverapamil in blood plasma or serum. Journal of Chromatography, **218**, 621-629.

Cole, S.P. (1986) Rapid chemosensitivity testing of human lung tumor cells using the MTT assay. Cancer Chemotherapy and Pharmacology, **17**, 259-263.

Cole, S.P., Downes, H.F. & Slovak, M.L. (1989) Effect of calcium antagonists on the chemosensitivity of two multidrug-resistant human tumour cell lines which do not overexpress P-glycoprotein. British Journal of Cancer, **59**, 42-46.

Cornwell, M.M., Gottesman, M.M. & Pastan, I.H. (1986A) Increased vinblastine binding to membrane vesicles from multidrug-resistant KB cells. Journal of Biological Chemistry, **261**, 7921-7928.

Cornwell, M.M., Safa, A.R., Felsted, R.L. Gottesman, M.M. & Pastan, I. (1986B) Membrane vesicles from multidrug-resistant human cancer cells contain a specific 150-to 170- kDa protein detected by photoaffinity labeling. Proceedings of the National Academy of Sciences of the United States of America, **83**, 3847-3850.

Cornwell, M.M., Pastan, I. & Gottesman, M.M. (1987) Certain calcium channel blockers bind specifically to multidrug-resistant human KB carcinoma membrane vesicles and inhibit drug binding to P-glycoprotein. Journal of Biological Chemistry, **262**, 2166-2170.

Cowan, K.H., Batist, G., Tulpule, A., Sinha, B.K. & Myers, C.E. (1986) Similar biochemical changes associated with multidrug resistance in human breast cancer cells and carcinogen-induced resistance to xenobiotics in rats. Proceedings of the National Academy of Sciences of the United States of America, **83**, 9328-9332.

Curtis, M.J. & Walker, M.J. (1986) The mechanism of action of the optical enantiomers of verapamil against ischaemia-induced arrhythmias in the conscious rat. British Journal of Pharmacology, **89**, 137-147.

Cuttitta, F., Rosen, S., Gazdar, A.F. & Minna, J.D. (1981) Monoclonal antibodies that demonstrate specificity for several types of human lung cancer. Proceedings of the National Academy of Sciences of the United States of America, **78**, 4591-4595.

Cuttitta, F., Carney, D.N., Mulshine, J., Moody, T.W., Fedorko, J., Fischler, A. & Minna, J.D. (1985) Bombesin-like peptides can function as autocrine growth factors in human small-cell lung cancer. Nature, **316**, 823-826.

Dalmark, M. & Storm, H.H. (1981) A Fickian diffusion transport process with features of transport catalysis. Doxorubicin transport in human red blood cells. Journal of General Physiology, **78**, 349-64.

Dalton, W.S., Cress, A.E., Alberts, D.S. & Trent, J.M. (1988) Cytogenetic and phenotypic analysis of a human colon carcinoma cell line resistant to mitoxantrone. Cancer Research, **48**, 1882-1888.

Dalton, W.S., Grogan, T.M., Meltzer, P.S., Scheper, R.J., Durie, B.G., Taylor, C.W., Miller, T.P. & Salmon, S.E. (1989) Drug-resistance in multiple myeloma and non-Hodgkin's lymphoma: detection of P-glycoprotein and potential circumvention by addition of verapamil to chemotherapy. Journal of Clinical Oncology, **7**, 415-424.

Danks, M.K., Yalowich, J.C. & Beck, W.T. (1987) Atypical multiple drug resistance in a human leukemic cell line selected for resistance to teniposide (VM-26). Cancer Research, **47**, 1297-1301.

De Bruijn, M.H., van der Bliek, A.M., Biedler, J.L. & Borst, P. (1986) Differential amplification and disproportionate expression of five genes in three multidrug-resistant Chinese hamster lung cell lines. Molecular and Cellular Biology, **6**, 4717-4722.

De Coursey, T.E., Chandy, K.G., Gupta, S. & Cahalan, M.D. (1985) Voltage-dependent ion channels in T-lymphocytes. Journal of Neuroimmunology, **10**, 71-95.

Deffie, A.M., Batra, J.K. & Goldenberg, G.J. (1989) Direct correlation between DNA topoisomerase II activity and cytotoxicity in adriamycin-sensitive and -resistant P388 Leukemia cell lines. Cancer Research, **49**, 58-62.

De Leij, L., Poppema, S., Nulend, J.K., Ter Haar, J.G., Schwander, E. & The, T.H. (1984) Immunoperoxidase staining on frozen tissue sections as a first screening assay in the preparation of monoclonal antibodies directed against small cell carcinoma of the lung. European Journal of Cancer and Clinical Oncology, **20**, 123-128.

De Leij, L., Postmus, P.E., Buys, C.H., Elema, J.D., Ramaekers, F., Poppema, S., Brouwer, M., van der Veen, A.Y., Mesander, G. & The, T.H. (1985) Characterization of three new variant type cell lines derived from small cell carcinoma of the lung. Cancer Research, **45**, 6024-6033.

Denizot, F. & Lang, R. (1986) Rapid colorimetric assay for cell growth and survival. Modifications to this tetrazolium dye procedure giving improved sensitivity and reliability. Journal of Immunological Methods, **89**, 271-277.

Deuchars, K.L., Du, R.P., Naik, M., Evernden-Porelle, D., Kartner, N., van der Bliek, A.M. & Ling, V. (1987) Expression of hamster P-glycoprotein and multidrug resistance in DNA-mediated transformants of mouse LTA cells. Molecular and Cellular Biology, **7**, 718-724.

Di Paolo, J.A. (1965) In vitro test systems for cancer chemotherapy. 3 : Preliminary studies of spontaneous mammary tumors in mice. Cancer Chemotherapy Reports, **44**, 19-24.

Dorr, R.T., Liddil, J.D., Trent, J.M. & Dalton, W.S. (1987) Mitomycin C resistant L1210 leukemia cells: association with pleiotropic drug resistance. Biochemical Pharmacology, **36**, 3115-20.

Douple, E.B., Cate, C.C., Curphey, T.J., Pettengill, O.S., Sorenson, G.D. & Maurer, L.H. (1985) Evaluation of Drug Efficacy In Vitro Using Human Small Cell Carcinoma of the Lung Spheroids. Cancer, **56**, 1918-1925.

Drewinko, B., Patchen, M., Yang, L.Y. & Barlogie, B. (1981) Differential killing efficacy of twenty antitumor drugs on proliferating and non-proliferating human tumor cells. Cancer Research, **41**, 2328-2333.

Duchesne, G.M., Eady, J.J., Peacock, J.H. & Pera, M.F. (1987) A panel of human lung carcinoma lines: establishment, properties and common characteristics. British Journal of Cancer, **56**, 287-293.

Dulik, D.M., Fenselau, C. & Hilton, J. (1986) Characterization of melphalan-glutathione adducts whose formation is catalysed by glutathione transferases. Biochemical Pharmacology, **35**, 3405-3409.

Dumont, P., van der Esch, E.P., Jabri, M., Lejeune, F. & Atassi, G. (1984) Chemosensitivity of human melanoma xenografts in immunocompetent mice and its histological evaluation. International Journal of Cancer, **33**, 447-451.

Dunnill, M.S. & Gatter, K.C. (1986) Cellular heterogeneity in lung cancer. Histopathology, **10**, 461-475.

Durkin, W.J., Ghanta, V.K., Balch, C.M., Davis, D.W. & Hiramoto, R.N. (1978) A methodological approach to the prediction of anti-cancer drug effect in humans. Cancer Research, **39**, 402-407.

Echizen, H., Brecht, T., Niedergesass, S., Vogelgesang, B. & Eichelbaum, M. (1985) The effect of dextro-, levo-, and racemic verapamil on atrioventricular conduction in humans. American Heart Journal, **109**, 210-217.

Endicott, J.A., Juranka, P.F., Sarangi, F., Gerlach, J.H., Deuchars, K.L. & Ling, V. (1987) Simultaneous expression of two P-glycoprotein genes in drug-sensitive Chinese hamster ovary cells. Molecular and Cellular Biology, **7**, 4075-4081.

Engelholm, S.A., Spang-Thomsen, M., Vindelov, L.L., Brunner, N., Nielsen, M.H., Hirsch, F., Nielson, A. & Hansen, H.H. (1986) Comparison of characteristics of human small cell carcinoma of the lung in patients, in vitro and transplanted into nude mice. Acta Pathologica Microbiologica et Immunologica Scandinavica Section A Pathology, **94**, 325-336.

Epstein, P.M., Fiss, K., Hachisu, R. & Andrenyak, D.M. (1982) Interaction of calcium antagonists with cyclic AMP phosphodiesterases and calmodulin. Biochemical and Biophysical Research Communications, **105**, 1142-1149.

Fairchild, C.R., Ivy, S.P., Kao-Shan, C.S., Whang-Peng, J., Rosen, N., Israel, M.A., Melera, P.W., Cowan, K.H. & Goldsmith, M.E. (1987) Isolation of amplified and over-expressed DNA sequences from adriamycin-resistant human breast cancer cells. Cancer Research, **47**, 5141-5148.

Falor, W.H., Ward-Skinner, R. & Wegryn, S. (1985) A 3p deletion in small cell lung carcinoma. Cancer Genetics and Cytogenetics, **16**, 175-177.

Farber, S., Diamond, L.K. & Mercer, R.P. (1948) Temporary remissions in acute leukemia in children produced by the folic acid antagonist, 4 aminopteroyl-glutamic acid (aminopterin). New England Journal of Medicine, **238**, 787-793.

Ferguson, P.J., Fisher, M.H., Stephenson, J., Li, D.H., Zhou, B.S. & Cheng, Y.C. (1988) Combined modalities of resistance in etoposide-resistant human KB cell lines. Cancer Research, **48**, 5956-5964.

Fergusson, R.J., Carmichael, J. & Smyth, J.F. (1986) Human tumour xenografts growing in immunodeficient mice: a useful model for assessing chemotherapeutic agents in bronchial carcinoma. Thorax, **41**, 376-380.

Ferry, D.R., Glossmann, H. & Kaumann, A.J. (1985) Relationship between the stereoselective negative inotropic effects of verapamil enantiomers and their binding to putative calcium channels in human heart. British Journal of Pharmacology, **84**, 811-824.

Fidler, I.J. & Kripke, M.L. (1977) Metastasis results from pre-existing variant cells within a malignant tumor. Science, **197**, 893-895.

Fidler, I.J. (1978) Tumor heterogeneity and the biology of cancer invasion and metastasis. Cancer Research, **38**, 2651-2660.

Figueredo, A., Arnold, A., Goodyear, M., Findlay, B., Neville, A., Normandeau, R. & Jones, A. (1990) Addition of Verapamil and Tamoxifen to the Initial Chemotherapy of Small Cell Lung Cancer. A Phase I/II Study. Cancer, **65**, 1895-1902.

Fine, R.L., Koizumi, S., Curt, G.A. & Chabner, B.A. (1987) Effect of calcium channel blockers on human CFU-GM with cytotoxic drugs. Journal of Clinical Oncology, **5**, 489-495.

Fitzgerald, D.J., Willingham, M.C., Cardarelli, C.O., Hamada, H., Tsuruo, T., Gottesman, M.M. & Pastan, I. (1987) A monoclonal antibody-Pseudomonas toxin conjugate that specifically kills multidrug-resistant cells. Proceedings of National Academy of Sciences of the United States of America, **84**, 4288-4292.

Fojo, A., Akiyama, S., Gottesman, M.M. & Pastan, I. (1985) Reduced drug accumulation in multiply drug-resistant human KB carcinoma cell lines. Cancer Research, **45**, 3002-3007.

Fojo, A.T., Shen, D.W., Mickley, L.A., Pastan, I. & Gottesman, M.M. (1987A) Intrinsic drug resistance in human kidney cancer is associated with expression of a human multidrug-resistance gene. Journal of Clinical Oncology, **5**, 1922-1927.

Fojo, A.T., Ueda, K., Slamon, D.J., Poplack, D.G., Gottesman, M.M. & Pastan, I. (1987B). Expression of a multidrug-resistance gene in human tumors and tissues. Proceedings National Academy of Sciences of the United States of America, **84**, 265-269.

Freshney, R.I., Paul, J. & Kane, I.M. (1975) Assay of anti-cancer drugs in tissue culture: conditions affecting their ability to incorporate ^3H -leucine after drug treatment. British Journal of Cancer, **31**, 89-99.

Frishman, W., Kirsten, E., Klein, M., Pine, M., Johnson, S.M., Hillis, L.D., Packer, M. & Kates, R. (1982) Clinical relevance of verapamil plasma levels in stable angina pectoris. American Journal of Cardiology, **50**, 1180-1184.

Gabutti, V., Pileri, A., Tarocco, R.P. & Gravosto, F. (1969) Proliferative potential of out-of-cycle leukaemic cells. Nature, **224**, 375-376.

Ganapathi, R., Grabowski, D., Turinic, R. & Valenzuela, R. (1984) Correlation between potency of calmodulin inhibitors and effects on cellular levels and cytotoxic activity of doxorubicin (adriamycin) in resistant P388 mouse leukemia cells. European Journal of Cancer and Clinical Oncology, **20**, 799-806.

Ganapathi, R. & Grabowski, D. (1988) Differential effect of the calmodulin inhibitor trifluoperazine in modulating cellular accumulation, retention and cytotoxicity of doxorubicin in progressively doxorubicin-resistant L1210 mouse leukaemia cells. Lack of correlation between cellular doxorubicin levels and expression of resistance. Biochemical Pharmacology, **37**, 185-193.

Gazdar, A.F., Carney, D.N., Russell, E.K., Sims, H.L., Baylin, S.B., Bunn, Jr., P.A., Guccion, J.G. & Minna, J.D. (1980) Establishment of continuous clonable cultures of small-cell carcinoma of lung which have amine precursor uptake and decarboxylation cell properties. Cancer Research, **40**, 3502-3507.

Gazdar, A.F., Carney, D.N. & Minna, J.D. (1983) The biology of non-small cell lung cancer. Seminars in Oncology, **10**, 3-19.

Gazdar, A.F., Carney, D.N., Nau, M.M. & Minna, J.D. (1985) Characterization of variant subclasses of cell lines derived from small cell lung cancer having distinctive biochemical, morphological and growth properties. Cancer Research, **45**, 2924-2930.

Gazdar, A.F. & Oie, H.K. (1986) Cell culture methods for human lung cancer. Cancer Genetics and Cytogenetics, **19**, 5-10.

Gerlach, J.H., Endicott, J.A., Juranka, P.F., Henderson, G., Sarangi, F., Deuchars, K.L. & Ling, V. (1986) Homology between P-glycoprotein and a bacterial haemolysin transport protein suggests a model for multidrug resistance. Nature, **324**, 485-489.

Gerlach, J.H., Bell, D.R., Karakousis, C., Slocum, H.K., Kartner, N., Rustum, Y.M., Ling, V. & Baker, R.M. (1987) P-glycoprotein in human sarcoma: evidence for multidrug resistance. Journal of Clinical Oncology, **5**, 1452-1460.

Glisson, B., Gupta, R., Hodges, P. & Ross, W. (1986) Cross-resistance to intercalating agents in an epipodophyllotoxin-resistant Chinese hamster ovary cell line: evidence for a common intracellular target. Cancer Research, **46**, 1939-1942.

Goldie, J.H. & Coldman, A.J. (1979) A mathematic model for relating the drug sensitivity of tumors to their spontaneous mutation rate. Cancer Treatment Reports, **63**, 1727-1733.

Goldstein, L.J., Galski, H., Fojo, A., Willingham, M., Lai, S.L., Gazdar, A., Pirker, R., Green, A., Crist, W., Brodeur, G.M., Grant, C., Lieber, M., Cossman, J., Gottesman, M.M. & Pastan, I. (1989) Expression of a multidrug resistance gene in human cancers. Journal of the National Cancer Institute, **81**, 116-124.

Goodman, G.E., Yen, Y.P., Cox, T.C. & Crowley, J. (1987) Effect of verapamil on in vitro cytotoxicity of adriamycin and vinblastine in human tumor cells. Cancer Research, **47**, 2295-2304.

Gottesman, M.M. & Pastan, I. (1988) The multidrug transporter, a double-edged sword. Journal of Biochemical Chemistry, **263**, 12163-12166.

Green, J.A., Vistica, D.T., Young, R.C., Hamilton, T.C., Rogan, A.M. & Ozols, R.F. (1984A) Potentiation of melphalan cytotoxicity in human ovarian cancer cell lines by glutathione depletion. Cancer Research, **44**, 5427-5431.

Green, L.M., Reade, J.L. & Ware, C.F. (1984B) Rapid colorimetric assay for cell viability: application to the quantitation of cytotoxic and growth inhibitory lymphokines. Journal of Immunological Methods, **70**, 257-268.

Gros, P., Ben Neriah, Y.B., Croop, J.M. & Housman, D.E. (1986A) Isolation and expression of a complementary DNA that confers multidrug resistance. Nature, **323**, 728-731.

Gros, P., Croop, J. & Housman, D. (1986B) Mammalian multidrug resistance gene: complete cDNA sequence indicates strong homology to bacterial transport proteins. Cell, **47**, 371-380.

Gullick, W.J., Marsden, J.J., Whittle, N., Ward, B., Bobrow, L. & Waterfield, M.D. (1986) Expression of epidermal growth factor receptors on human cervical, ovarian and vulval carcinomas. Cancer Research, **46**, 285-292.

Hamada, H., Hagiwara, K.I., Nakajima, T. & Tsuruo, T. (1987) Phosphorylation of the M 170,000 to 180,000 glycoprotein specific to multidrug-resistant tumor cells: effects of verapamil, trifluoperazine and phorbol esters. Cancer Research, **47**, 2860-2865.

Hamburger, A.W. & Salmon, S.E. (1977) Primary bioassay of human tumor stem cells. Science, **197**, 461-463.

Hamburger, A.W., Salmon, S.E., Kim, M.B., Trent, J.M., Soehnlen, B.J., Alberts, D.S. & Schmidt, H.J. (1978) Direct cloning of human ovarian carcinoma cells in agar. Cancer Research, **38**, 3438-3444.

Hamburger, A.W. (1981) Use of in vitro tests in predictive cancer chemotherapy. Journal of the National Cancer Institute, **66**, 981-988.

Hamburger, A. W., White, C.P. & Tencer, K. (1982) Effect of enzymatic disaggregation on proliferation of human tumor cells in soft agar. Journal of the National Cancer Institute, **68**, 945-949.

Hamilton, T.C., Winter, M.A., Louie, K., Batist, G., Behrens, B.C., Tsuruo, T., Grotzinger, K.R., McKoy, W.M., Young, R.C. & Ozols, R.F. (1985) Augmentation of adriamycin, melphalan and cisplatin cytotoxicity in drug-resistant and -sensitive human ovarian carcinoma cell lines by buthionine sulfoximine mediated glutathione depletion. Biochemical Pharmacology, **34**, 2583-2586.

Hansen, H.H. (1987) Chemotherapy of small cell carcinoma: a review. Quarterly Journal of Medicine, **63**, 275-282.

Harbour, J.W., Lai, S., Whang-Peng, J., Gazdar, A.F., Minna, J.D. & Kaye, F.J. (1988) Abnormalities in structure and expression of the human retinoblastoma gene in SCLC. Science, **241**, 353-357.

Harker, W.G., Bauer, D., Etiz, B.B., Newman, R.A. & Sikic, B.I. (1986) Verapamil-mediated sensitization of doxorubicin-selected pleiotropic resistance in human sarcoma cells: selectivity for drugs which produce DNA scission. Cancer Research, **46**, 2369-2373.

Harrow, J.A. & Dhalla, N.S. (1976) Effects of quinidine on calcium transport activities of the rabbit heart mitochondria and sarcotubular vesicles. Biochemical Pharmacology, **25**, 897-902.

Hayashi, I. & Sato, G. (1976) Replacement of serum by hormones permits growth of cells in a defined medium. Nature, **259**, 132-134.

Hecht, H.S., Chew, C.Y., Burnam, M.H., Hopkins, J., Schnugg, S. & Singh, B.N. (1981) Verapamil in chronic stable angina: amelioration of pacing-induced abnormalities of left ventricular ejection fraction, regional wall motion, lactate metabolism and hemodynamics. American Journal of Cardiology, **48**, 536-544.

Heeg, K., Reimann, J., Kabelitz, D., Hardt, C. & Wagner, H. (1985) A rapid colorimetric assay for the determination of IL-2-producing helper T cell frequencies. Journal of Immunological Methods, **77**, 237-246.

Herrmann, E.C., Gabliks, J., Engle, C. & Perlman, P.L. (1960) Agar diffusion method for detection and bioassay of anti-viral antibiotics. Proceedings of the Society for Experimental Biology and Medicine, **103**, 625-628.

Hess, R. & Pearse, G.E. (1963) Pathways of reduced pyridine nucleotide oxidation in rat-brain homogenate demonstrated by a tetrazolium method. Biochemica et Biophysica Acta, **71**, 285-294.

Hill, B.T. (1983) Use of continuous human tumour cell lines to evaluate drugs by clonogenic assays. In Human Tumour Drug Sensitivity Testing In Vitro: techniques and clinical applications. ed. Dendy, P.P. & Hill, B.T. pp 129. London: Academic Press.

Hirsch, F.R., Ottesen, G., Podenphant, J. & Olsen, J. (1983) Tumour heterogeneity in lung cancer based on light microscopic features: a retrospective study of 200 patients treated surgically. Virchows Archiv A. Pathological Anatomy and Histopathology, **42**, 147-153.

Holleran, W.M., DeGregorio, M.W., Ganapathi, R., Wilbur, J.R. & Macher, B.A. (1986) Characterization of cellular lipids in doxorubicin-sensitive and -resistant P388 mouse leukemia cells. Cancer Chemotherapy and Pharmacology, **17**, 11-15.

Horie, A. & Ohta, M. (1981) Ultrastructural features of large cell carcinoma of lung with reference to the prognosis of patients. Human Pathology, **12**, 423-432.

Horio, M., Gottesman, M.M. & Pastan, I. (1988) ATP-dependent transport of vinblastine in vesicles from human multidrug-resistant cells. Proceedings of the National Academy of Sciences of the United States of America, **85**, 3580-3584.

Hug, V., Thames, H., Haynes, M., Blumenschein, G., Drewinko, B. & Spitzer, G. (1984) Use of normalized drug sensitivities of human breast tumours for clinical correlations and for comparison of drug activities. In Human Tumor Cloning. ed. Salmon, S.E. & Trent, J.M. pp 563. New York: Grune & Stratton.

Iglehart, J.D., Wolfe, W.G., Vernon, W.B., Shelburne, J.D. & Sabiston, D.C. (1985) Electron microscopy in selection of patients with small cell carcinoma of the lung for medical versus surgical therapy. Journal of Thoracic and Cardiovascular Surgery, **90**, 351-360.

Ihde, D.C. (1984) Current status of therapy for small cell carcinoma of the lung. Cancer, **54**, (II Suppl) 2722-2728.

Inaba, M. & Johnson, R.K. (1978) Uptake and retention of adriamycin and daunorubicin by the sensitive and anthracycline resistant sublines of P388 leukemia. Biochemical Pharmacology, **27**, 2123-2130.

Inaba, M., Kobayashi, H., Sakurai, Y. & Johnson, R.K. (1979) Active efflux of daunorubicin and adriamycin in sensitive and resistant sublines of P388 leukemia. Cancer Research, **39**, 2200-2203.

Isaacs, J.T. & Coffey, D.S. (1981) Adaptation versus selection as the mechanism responsible for the relapse of prostatic cancer to androgen ablation therapy as studied in the Dunning R-3327-H adenocarcinoma. Cancer Research, **41**, 5070-5075.

Jakoby, W.B. (1978) The glutathione-S-transferases: a group of multifunctional detoxification proteins. Advances in Enzymology and Related Areas of Molecular Biology, **46**, 383-414.

Janis, R.A. & Scriabine, A. (1983) Sites of action of Ca^{2+} channel inhibitors. Biochemical Pharmacology, **32**, 3499-3507.

Jones, A.C., Stratford, I.J., Wilson, P.A. & Peckham, M.J. (1982) In Vitro Cytotoxic Drug Sensitivity Testing of Human Tumour Xenografts grown as Multicellular Tumour Spheroids. British Journal of Cancer, **46**, 870-879.

Jongsma, A.P., Spengler, B.A., Van der Bliek, A.M., Borst, P. & Biedler, J.L. (1987) Chromosomal localization of three genes coamplified in the multidrug-resistant CHRC5 Chinese hamster ovary cell line. Cancer Research, **47**, 2875-2878.

Kaelin, W.G., Jr., Scrivastav, S., Shand, D.G. & Jirtle, R.L. (1982) Effect of Verapamil on malignant tissue blood flow in SMT-2A tumor bearing rats. Cancer Research, **42**, 3944-3949.

Kahan, B.D., Ried, M. & Newburger, J. (1983) Pharmacokinetics of cyclosporin in human renal transplantation. Transplantation Proceedings, **15**, 446-453.

Kahn, E., Bernard, J. & DiPaola, R. (1986) The use of an image analyzer in human tumour clonogenic assays. Cytometry, **7**, 313-317.

Kaltenbach, J.P., Kaltenbach, M.H. & Lyons, W.B. (1958) Nigrosin as a dye for differentiating live and dead ascites cells. Experimental Cell Research, **15**, 112-117.

Kartner, N., & Ling, V. (1989) Multidrug resistance in cancer. Scientific American, **260**, 26-33.

Kemp, I., Boyle, P., Smans, M. & Muir, C. (1985) In Atlas of Cancer in Scotland, 1975-1980. Incidence and Epidemiological Perspective. I.A.R.C. Scientific Publication No. 72. Lyon: International Agency for Research on Cancer.

Kerbel, R.S. (1979) Implications of immunological heterogeneity of tumours. Nature, **280**, 358-360.

Kerr, D.J., Graham, J., Cummings, J., Morrison, J.G., Thompson, G.G., Brodie, M.J. & Kaye, S.B. (1986) The effect of verapamil on the pharmacokinetics of adriamycin. Cancer Chemotherapy and Pharmacology, **18**, 239-242.

Kessel, D. & Wilberding, C. (1985) Anthracycline resistance in P388 murine leukaemia and its circumvention by calcium antagonists. Cancer Research, **45**, 1687-1691.

Klohs, W.D. & Steinkampf, R.W. (1988) Possible link between the intrinsic drug resistance of colon tumors and a detoxification mechanism of intestinal cells. Cancer Research, **48**, 3025-3030.

Kodate, C., Fukushi, A., Narita, T., Kuda, H., Soma Y. & Sato, K. (1986) Human placental form of glutathione S-transferase (GST-pi) as a new immuno-histochemical marker for human colonic carcinoma.

Japanese Journal of Cancer Research, **77**, 226-229.

Kohlhardt, M., Bauer, P., Krause, H. & Fleckenstein, A. (1972) Differentiation of the transmembrane Na and Ca channels in mammalian cardiac fibres by the use of specific inhibitors. Pfluegers Archiv European Journal of Physiology, **335**, 309-322.

Kok, K., Osinga, J., Carritt, B., Davis, M.B., van der Hout, A.H., van der Veen, A.Y., Landsvater, R.M., De Leij, L.F., Berendsen, H.H., Postmus, P.E., Poppema, S. & Buys, C.H. (1987) Deletion of a DNA sequence at the chromosomal region 3p 21 in all major types of lung cancer. Nature, **330**, 578-581.

Kondo, T. & Ohkubo, K. (1967) In vitro test for prediction of side-effects of carcinostatic agents. Gann: Japanese Journal of Cancer Research, **58**, 349-354.

Kornblith, P.L. & Szypko, P.E. (1978) Variations in response of human brain tumors to BCNU in vitro. Journal of Neurosurgery, **48**, 580-586.

Kramer, R.A., Greene, K., Ahmad, S. & Vistica, I. (1987) Chemosensitization of L-phenylalanine mustard by the thiol-modulating agent buthionine sulfoximine. Cancer Research, **47**, 1593-1597.

Kuratomi, Y., Akiyama, S., Ono, M., Shiraishi, N., Shimada, R., Ohkuma, S. & Kuwano, M. (1986) Thioridazine enhances lysosomal accumulation of epidermal growth factor and toxicity of conjugates of epidermal growth factor with *Pseudomonas* exotoxin. Experimental Cell Research, **162**, 436-448.

Kwok, T.T. & Twentyman, P.R. (1987) Use of a tritiated thymidine suicide technique in the study of the cytotoxic drug response of cells located at different depths within multicellular spheroids. British Journal of Cancer, **55**, 367-374.

Labarca, C. & Paigen, K. (1980) A simple, rapid and sensitive DNA assay procedure. Analytical Biochemistry, **102**, 344-352.

Lai, S.L., Goldstein, L.J., Gottesman, M.M., Pastan, I., Tsai, C.M., Johnson, B.E., Mulshine, J.L., Ihde, D.C., Kayser, K. & Gazdar, A.F. (1989) MDRI gene expression in lung cancer. Journal of the National Cancer Institute, **81**, 1144-1150.

Laszlo, J., Stengle, J., Wight, K. & Burk, D. (1958) Effects of chemotherapeutic agents on metabolism of human acute leukemia cells in vitro. Proceedings of the Society for Experimental Biology and Medicine, **97**, 127-131.

Law, L. W. (1952) Origin of the resistance of leukaemic cells to folic acid antagonists. Nature, **169**, 628-629.

Lehto, V.P., Stenman, S., Miettinen, M.N., Dahl, D. & Virtanen, I. (1983) Expression of a neural type of intermediate filament as a distinguishing feature between oat cell carcinoma and other lung cancers. American Journal of Pathology, **110**, 113-118.

Leu, R.W. & Herriott, M.J. (1984) Microassay for photometric quantitation of macrophage mediated tumor cytotoxicity using an automated densitometer. Journal of Immunological Methods, **67**, 63-72.

Levi, F.A., Blum, J.P., Lemaigre, G., Bourot, C., Reinberg, A. & Mathe, G. (1984) A four-day sub-renal capsule assay for testing the effectiveness of anticancer drugs against human tumors. Cancer Research, **44**, 2660-2667.

Ling, V. & Thompson, L.H. (1974) Reduced permeability in CHO cells as a mechanism of resistance to colchicine. Journal of Cell Physiology, **83**, 103-116.

Livingston, R.B., Titus, G.A. & Heilbrun, L.K. (1980) In vitro effects on DNA synthesis as a predictor of biological effect from chemotherapy. Cancer Research, **40**, 2209-2212.

Lotan, R. & Nicolson, G.L. (1979) Heterogeneity in growth inhibition by beta-trans-retinoic acid of metastatic B16 melanoma clones and in vivo - selected cell variant lines. Cancer Research, **39**, 4767-4771.

Louie, K.G., Hamilton, T.C., Winker, M.A., Behrens, B.C., Tsuruo, T., Klecker, R.W., McKoy, W.M., Grotzinger, K.R., Myers, C.E. & Young, R.C. (1986) Adriamycin accumulation and metabolism in adriamycin - sensitive and - resistant human ovarian cancer cell lines. Biochemical Pharmacology, **35**, 467-472.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951) Protein measurement with the folin phenol reagent. Journal of Biological Chemistry, **193**, 265-275.

Luria, S.E. & Delbruck, M. (1943) Mutations of bacteria from virus sensitivity to virus antagonists. Genetics, **28**, 491-511.

McDowell, E.M. & Trump, B.E. (1981) Pulmonary small cell carcinoma showing tripartite differentiation in individual cells. Human Pathology, **12**, 286-294.

Mackillop, W.J., Ciampi, A., Till, J.E. & Buick, R.N. (1983) A stem cell model of human tumor growth : implications for tumor cell clonogenic assays. Journal of the National Cancer Institute, **70**, 9-16.

Ma, D.D., Scurr, R.D., Davey, R.A., Mackerhich, S.M., Harman, D.H., Dowden, G., Isbister, J.P. & Bell, D.R. (1987) Detection of a multidrug resistant phenotype in acute non-lymphoblastic leukaemia. Lancet, **1**, 135-137.

Makin, C.A., Bobrow, L.G. & Bodmer, W.F. (1984) Monoclonal antibody to cytokeratin for use in routine histopathology. Journal of Clinical Pathology, **37**, 975-983.

Manoharan, T.H., Puchalski, R.B., Burgess, J.A., Pickett, C.B. & Fahl, W.E. (1987) Promoter-glutathione S-transferase Ya cDNA hybrid genes. Expression and conferred resistance to an alkylating molecule in mammalian cells. Journal of Biological Chemistry, **262**, 3739-3745.

Marangos, P.J., Gazdar, A.F. & Carney, D.N. (1982) Neuron specific enolase in human small cell carcinoma cultures. Cancer Letters, **15**, 67-71.

Marsh, W. & Center, M.S. (1987) Adriamycin resistance in HL60 cells and accompanying modification of a surface membrane protein contained in drug-sensitive cells. Cancer Research, **47**, 5080-5086.

Matsushima, Y., Kanzawa, F., Hoshi, A., Shimizu, E., Nomori, H., Sasaki, Y. & Saijo, N. (1985) Time-schedule dependency of the inhibiting activity of various anti-cancer drugs in the clonogenic assay. Cancer Chemotherapy and Pharmacology, **14**, 104-107.

Matthews, M.J. & Hirsch, F.R. (1981) Problems in the Diagnosis of small cell carcinoma of the lung. In Small Cell Lung Cancer. ed. Greco, F.A., Oldham, R.K. & Bunn, P.A. Jr. pp 35-50. New York: Grune & Straton.

Mealey, J., Chen, T.T. & Shupe, R. (1974) Response of cultured human glioblastomas to radiation and BCNU chemotherapy. Journal of Neurosurgery, **41**, 339-349.

Meister, A. (1983) Selective modification of glutathione metabolism. Science, **220**, 472-477.

Merrill, W.W. & Bondy, P.K. (1982) Production of biochemical marker substances in bronchogenic cancers. Clinics in Chest Medicine, **3**, 307-320.

Miller, B.E., Miller, F.R. & Heppner, G.H. (1981) Interactions between tumor subpopulations affecting their sensitivity to the anti-neoplastic agents cyclophosphamide and methotrexate. Cancer Research, **41**, 4378-4381.

Miller, B.E., Miller, F.R. & Heppner, G.H. (1984) Assessing tumor drug sensitivity by a new in vitro assay which preserves tumor heterogeneity and subpopulation interactions. Journal of Cellular Physiology Supplement, **3**, 105-116.

Miller, R.L., Bukowski, R.M., Budd, G.T., Purvis, J., Weick, J.K., Shepard, K., Midha, K.K. & Ganapathi, R. (1988) Clinical modulation of doxorubicin resistance by the calmodulin-inhibitor trifluoperazine: a phase I/II trial. Journal of Clinical Oncology, **6**, 880-888.

Minna, J.D., Cuttitta, F., Rosen, S., Bunn, P.A., Carney, D.N., Gazdar, A.F. & Krasnow, S. (1981) Methods for production of monoclonal antibodies with specificity for human lung cancer cells. In Vitro, **17**, 1058-1070.

Minna, J.D., Carney, D.N., Oie, H., Bunn, Jr., P.A. & Gazdar, A.F. (1982) In Growth of Human small-cell lung cancer in defined medium. ed. Pardee, A. & Sato, G. Vol. 9, pp629-639. Cold Spring Harbor, New York: Cold Spring Harbor Press.

Mirski, S.E., Gerlach, J.H. & Cole, S.P. (1987) Multidrug resistance in human small cell lung cancer cell line selected in adriamycin. Cancer Research, **47**, 2594-2598.

Moody, T.W., Pert, C.B., Gazdar, A.F., Carney, D.N. & Minna, J.D. (1981) High levels of intracellular bombesin characterise human small-cell lung carcinoma. Science, **214**, 1246-1248.

Moody, T.W., Carney, D.N., Korman, L.Y., Gazdar, A.F. & Minna, J.D. (1985) Neurotensin is produced by and secreted from classic small cell lung cancer cells. Life Sciences, **36**, 1727-1732.

Mooi, W.J., Dingemans, K.P. & Van Zandwijk, N. (1986) Prevalence of neuroendocrine granules in small cell lung carcinoma. Usefulness of electron microscopy in lung cancer classification. Journal of Pathology, **149**, 41-47.

Morgan, D. & Freshney, R.I. (1979) In vitro predictive testing of astrocytoma. British Journal of Cancer, **40**, 303-304 (abstract).

Morgan, D., Freshney, R.I., Darling, J.L., Thomas, D.G. & Celik, F. (1983) Assay of anticancer drugs in tissue culture: cell cultures of biopsies from human astrocytoma. British Journal of Cancer, **47**, 205-214.

Morstyn, G., Ihde, D.C., Lichter, A.S., Bunn, P.A., Carney, D.N., Glatstein, E. & Minna, J.D. (1984) Small cell lung cancer 1973-1983 : early progress and recent obstacles. International Journal of Radiation Oncology, Biology and Physics, **10**, 515-539.

Moscow, J.A. & Cowan, K.H. (1988) Multidrug resistance. Journal of the National Cancer Institute, **80**, 14-20.

Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of Immunological Methods, **65**, 55-63.

Mostow, N.D., Rakita, L., Vrobel, T.R., Noon, D. & Blumer, J. (1984) Amiodarone: intravenous loading for rapid suppression of complex ventricular arrhythmias. Journal of the American College of Cardiology, **1**, 97-104.

Nagourney, R. (1989) Written communication.

Nakata, Y. & Bader, J.P. (1969) The uptake of nucleosides by cells in culture. II. Inhibition by 2-mercapto-1-(beta-4-pyridethyl) benzimidazole. Biochemica et Biophysica Acta, **130**, 250-256.

Nederman, T. & Twentyman, P. (1984) Spheroids for studies of drug effects. Recent Results in Cancer Research, **95**, 84-102.

Newrath, H., Blei, I., Gegner, A., Ludwig, C. & Zong, X.G. (1981) No stereoselective effects of the optical isomers of verapamil and D-600 in the heart. In Calcium antagonists in cardiovascular therapy: experience with Verapamil. ed. Zanchetti, A. & Krikler, D.M. pp 52-63. Amsterdam: Excerpta Medica.

Nishimura, T., Suzuki, H., Muto, K. & Tanaka, N. (1979) Mechanism of adriamycin resistance in a subline of mouse lymphoblastoma L5178Y cells. Journal of Antibiotics, **32**, 518-522.

Oboshi, S., Tsugawa, S., Seido, T., Shimosato, Y., Koide, T. & Ishikawa, S. (1971) A new floating cell line derived from human pulmonary carcinoma of oat cell type. Gann: Japanese Journal of Cancer Research, **62**, 505-514.

O'Dwyer, P.J. (1989) Written communication.

Ohara, H. & Okamoto, T. (1977) A new in vitro cell line established from human oat cell carcinoma of the lung. Cancer Research, **37**, 3088-3095.

Ozols, R.F., Cunnion, R.E., Klecker, R.W., Hamilton, T.C., Ostchega, Y., Parrillo, J.E. & Young, R.C. (1987A) Verapamil and adriamycin in the treatment of drug resistant ovarian cancer patients. Journal of Clinical Oncology, **5**, 641-647.

Ozols, R.F., Louie, K.G., Plowman, J., Behrens, B.C., Fine, R.L., Dykes, D. & Hamilton, T.C. (1987B) Enhanced melphalan cytotoxicity in human ovarian cancer in vitro and in tumor-bearing nude mice by buthionine sulfoximine depletion of glutathione. Biochemical Pharmacology, **36**, 147-153.

Pastan, I., Gottesman, M.M., Ueda, K., Lovelace, E., Rutherford, A.V. & Willingham, M.C. (1988) A retrovirus carrying an MDRI cDNA confers multidrug resistance and polarized expression of P-glycoprotein in MDCK cells. Proceedings of the National Academy of Sciences of the United States of America, **85**, 4486-4490.

Pettengill, O.S., Faulkner, C.S., Wurster-Hill, D.H., Maurer, L.H., Sorenson, G.D., Robinson, A.G. & Zimmerman, E.A. (1977) Isolation and characterisation of a hormone-producing cell line from human small cell anaplastic carcinoma of the lung. Journal of the National Cancer Institute, **58**, 511-518.

Pettengill, O.S., Sorensen, G.D., Wurster-Hill, D.H., Curphey, T.J., Noll, W.W., Cate, C.C. & Maurer, L.H. (1980) Isolation and growth characteristics of continuous cell lines from small-cell carcinoma of the lung. Cancer, **45**, 906-918.

Pettengill, O.S., Wurster-Hill, D.H., Cate, C.C. & Sorenson, G.D. (1985) Morphological growth characteristics and hormone secretion of small cell carcinoma of the lung in vitro. Recent Results in Cancer Research, **99**, 130-142.

Plumb, J.A., Milroy, R. & Kaye, S.B. (1989) Activity of resistance modifiers in lung cancer cell lines. Proceedings of the American Association for Cancer Research, **30**, 572 (abstract).

Pommier, Y., Kerrigan, D., Schwartz, R., Swack, J.A. & McCurdy, A. (1986) Altered DNA topoisomerase II activity in Chinese hamster cells resistant to topoisomerase II inhibitors. Cancer Research, **46**, 3075-3081.

Poste, G. & Fidler, I.J. (1980) The pathogenesis of cancer metastasis. Nature, **283**, 139-146.

Postmus, P.E., de Ley, L., van der Veen, A.Y., Mesander, G., Buys, C.H. & Elema, J.D. (1988) Two small cell lung cancer cell lines established from rigid bronchoscopic biopsies. European Journal of Cancer and Clinical Oncology, **24**, 753-763.

Praet, M., Laghmiche, M., Pollakis, G., Goormaghtigh, E. & Ruysschaert, J.M. (1986) In vivo and in vitro modifications of the mitochondrial membrane induced by 4'Epi-adriamycin. Biochemical Pharmacology, **35**, 2923-2928.

Presant, C.A., Kennedy, P.S., Wiseman, C., Gala, K., Bouzaglou, A., Wyres, M. & Naessig, V. (1986) Verapamil reversal of clinical doxorubicin resistance in human cancer. A Wilshire Oncology Medical Group pilot phase I-II study. American Journal of Clinical Oncology, **9**, 355-357.

Radel, S., Bankusli, I., Mayhew, E. & Rustum, Y.M. (1988) The effects of verapamil and a tiapamil analogue, DMDP, on adriamycin-induced cytotoxicity in P388 adriamycin-resistant and-sensitive leukemia in vitro and in vivo. Cancer Chemotherapy and Pharmacology, **21**, 25-30.

Raich, P.C. (1978) Prediction of therapeutic response in acute leukaemia. Lancet, **2**, 74-76.

Raikhlin, N.T., Warzok, R. & Smirnowa, E.A. (1979) Zur Histogenese des kleinzelligen Bronchialkarzinoms. Zentralblatt fur Allgemeine Pathologie und Pathologische Anatomie, **123**, 202-208.

Rainio, P., Sutinen, S. & Sutinen, S.H. (1983) Histological subtypes or grading of pulmonary adenocarcinoma. A histochemical and electron microscopic study. Acta Pathologica Microbiologica et Immunologica Scandinavica Section A Pathology, **91**, 227-234.

Ramu, A., Glaubiger, D., Magrath, I.T. & Joshi, A. (1983) Plasma membrane lipid structural order in doxorubicin-sensitive and -resistant P388 cells. Cancer Research, **43**, 5533-5537.

Ramu, A., Spanier, R., Rahamimoff, H. & Fuks, Z. (1984) Restoration of doxorubicin responsiveness in doxorubicin-resistant P388 murine leukaemia cells. British Journal of Cancer, **50**, 501-507.

Reiter, M.J., Shand, D.G., Aanonsen, L.M., Wagoner, R., McCarthy, E. & Pritchett, E.L. (1982) Pharmacokinetics of verapamil: experiences with a sustained intravenous infusion regimen. American Journal of Cardiology, **52**, 716-721.

Reynolds, E.S. (1963) The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. Journal of Cell Biology, **17**, 208-212.

Riordan, J.R., Deuchars, K., Kartner, N., Alon, M., Trent, J. & Ling, V. (1985) Amplification of P-glycoprotein genes in multidrug resistant mammalian cell lines. Nature, **316**, 817-819.

Riordan, J.R. & Ling, V. (1985) Genetic and biochemical characterization of multidrug resistance. Pharmacology and Therapeutics, **28**, 51-75.

Rogan, A.M., Hamilton, T.C., Young, R.C., Klecker, R.W. & Ozols, R.F. (1984) Reversal of adriamycin resistance by verapamil in human ovarian cancer. Science, **224**, 994-996.

Roninson, I.B., Abelson, H.T., Housman, D.E., Howell, N. & Varshavsky, A. (1984) Amplification of specific DNA sequences correlates with multi-drug resistance in Chinese hamster cells. Nature, **309**, 626-628.

Roninson, I.B., Chin, J.E., Choi, K.G., Gros, P., Housman, D.E., Fojo, A., Shen, D.W., Gottesman, M.M. & Pastan, I. (1986) Isolation of human *mdr* DNA sequences amplified in multidrug-resistant KB carcinoma cells. Proceedings of the National Academy of Sciences of the United States of America, **83**, 4538-4542.

Rook, G.A., Steele, J., Umar, S. & Dockrell, H.M. (1985) A simple method for the solubilisation of reduced NBT and its use as a colorimetric assay for activation of human macrophages by gamma-interferon. Journal of Immunological Methods, **82**, 161-167.

Roper, P.R. & Drewinko, B. (1976) Comparison of in vitro methods to determine drug-induced cell lethality. Cancer Research, **36**, 2182-2188.

Roper, P.R. & Drewinko, B. (1979) Cell survival following treatment with antitumour drugs. Cancer Research, **39**, 1428-1430.

Rothenberg, M. & Ling, V. (1989) Multidrug resistance: molecular biology and clinical relevance. Journal of the National Cancer Institute, **81**, 907-910.

Rotman, B. & Papermaster, B.W. (1966) Membrane properties of living mammalian cells as studied by enzymatic hydrolysis of fluorogenic esters. Proceedings of the National Academy of Sciences of the United States of America, **55**, 134-141.

Ruben, R.L. & Neubauer, R.H. (1987) Semiautomated colorimetric assay for in vitro screening of anticancer compounds. Cancer Treatment Reports, **71**, 1141-1149.

Ruckdeschel, J.C., Carney, D.N., Oie, H.K., Russell, E.K. & Gazdar, A.F. (1987) In vitro chemosensitivity of human lung cancer cell lines. Cancer Treatment Reports, **71**, 697-704.

Rushmore, T.H., Sharma, R.N., Roomi, M.W., Harris, L., Satoh, K., Sato, K., Murray, R.K. & Farber, E. (1987) Identification of a charactersitic cytosolic polypeptide of rat pre-neoplastic hepatocyte nodules as placental glutathione-S-transferase. Biochemical and Biophysical Research Communications, **143**, 98-103.

Russo, A. & Mitchell, J.B. (1985) Potentiation and protection of doxorubicin cytotoxicity by cellular glutathione modulation. Cancer Treatment Reports, **69**, 1293-1296.

Saba, S.R., Espinoza, C.G., Richman, A.V. & Azar, H.A. (1983) Carcinomas of the lung: an ultrastructural and immuno-cytochemical study. American Journal of Clinical Pathology, **80**, 6-13.

Safa, A.R., Glover, C.J., Meyers, M.B., Biedler, J.L. & Felsted, R.L. (1986) Vinblastine photoaffinity labeling of a high molecular weight surface membrane glycoprotein specific for multidrug-resistant cells. Journal of Biological Chemistry, **261**, 6137-6140.

Salmon, S.E. & Von Hoff, D.D. (1981) In vitro evaluation of anti-cancer drugs with the human tumor stem cell assay. Seminars in Oncology, **8**, 377-385.

Sanfilippo, O., Silverstrini, R., Zaffaroni, N., Piva, L. & Pizzocaro, G. (1986) Application of an in vitro antimetabolic assay to human germ cell testicular tumors for the preclinical evaluation of drug sensitivity. Cancer, **58**, 1441-1447.

Sarosdy, M.F., Schneider, S.L., Forseth, B.J. & Zeltzer, P.M. (1986) In vitro chemosensitivity of human neuroblastoma cell lines in soft agar: a tumor specific drug screening model. Investigational New Drugs, **4**, 135-139.

Satoh, K., Kitahara, A., Soma, Y., Inaba, Y., Hatayama, I. & Sato, K. (1985) Purification, induction and distribution of placental glutathione transferase: a new marker enzyme for preneoplastic cells in the rat chemical hepatocarcinogenesis. Proceedings of the National Academy of Sciences of the United States of America, **82**, 3964-3968.

Saunders, E.F. & Mauer, A.M. (1969) Reentry of nondividing leukaemic cells into a proliferative phase in acute childhood leukaemia. Journal of Clinical Investigation, **48**, 1299-1305.

Saydjari, R., Townsend, C.M., Barranco, S.C., James, E. & Thompson, J.C. (1986) Effects of cyclosporin A and alpha-difluoromethylornithine on the growth of hamster pancreatic cancer in vitro. Journal of the National Cancer Institute, **77**, 1087-1092.

Schaeffer, W.I. & Friend, K. (1976) Efficient detection of soft-agar grown colonies using a tetrazolium salt. Cancer Letters, **1**, 275-279.

Scheithauer, W., Clark, G.M., Moyer, M.P. & Von Hoff, D.D. (1986) New screening system for selection of anticancer drugs for treatment of human colorectal cancer. Cancer Research, **46**, 2703-2708.

Schol, D.J., Mooi, W.J., van der Gugten, A.A., Wagenaar, S.S. & Hilgers, J. (1988) Monoclonal antibody 123C3 identifying small cell carcinoma phenotype in lung tumours recognizes mainly, but not exclusively endocrine and neuron-supporting normal tissues. International Journal of Cancer, (Suppl), **2**, 34-40.

Schuurhuis, G.J., Broxterman, H.J., van der Hoeven, J.J., Pinedo, H.M. & Lankelma, J. (1987) Potentiation of doxorubicin cytotoxicity by the calcium antagonist bepridil in anthracycline-resistant and-sensitive cell lines. A comparison with verapamil. Cancer Chemotherapy and Pharmacology, **20**, 285-290.

Scotto, K.W., Biedler, J.L. & Melera, P.W. (1986) Amplification and expression of genes associated with multidrug resistance in mammalian cells. Science, **232**, 751-755.

Scudiero, D.A., Shoemaker, R.H., Paull, K.D., Monks, A., Tierney, S., Nofziger, T.H., Currens, M.J., Seniff, D. & Boyd, M.R. (1988) Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other cell lines. Cancer Research, **48**, 4827-4833.

Seeber, S., Osieka, R., Schmidt, C.G., Achterrath, W. & Crooke, S.T. (1982) In vivo resistance towards anthracyclines, etoposide and cis-diaminedichloroplatinum (II). Cancer Research, **42**, 4719-4725.

Sehested, M., Skovsgaard, T., van Deurs, B. & Winther-Nielsen, H. (1987A) Increase in nonspecific adsorptive endocytosis in anthracycline- and vinca alkaloid-resistant Ehrlich ascites tumor cell lines. Journal of the National Cancer Institute, **78**, 171-179.

Sehested, M., Skovsgaard, T., van Deurs, B. & Winther-Nielsen, H. (1987B) Increased plasma membrane traffic in daunorubicin resistant P388 leukaemic cells. Effect of daunorubicin and verapamil. British Journal of Cancer, **56**, 747-751.

Seifter, E.J. & Ihde, D.C. (1988) Therapy of small cell lung cancer: a perspective on two decades of clinical research. Seminars in Oncology, **15**, 278-299.

Selby, P., Buick, R.N. & Tannock, I. (1983) A critical appraisal of the "human tumor stem-cell assay". New England Journal of Medicine, **308**, 129-134.

Selby, P. (1984) Acquired resistance to cancer chemotherapy. British Medical Journal, **288**, 1252-1253.

Shen, D.W., Cardarelli, C., Hwang, J., Cornwell, M., Richert, N., Ishii, S., Pastan, I. & Gottesman, M.M. (1986A) Multiple drug resistant human KB carcinoma cells independently selected for high-level resistance to colchicine, adriamycin or vinblastine show changes in expression of specific proteins. Journal of Biological Chemistry, **261**, 7762-7760.

Shen, D.W., Fojo, A., Chin, J.E., Roninson, I.B., Richert, N., Pastan, I. & Gottesman, M.M. (1986B) Human multidrug-resistant cell lines: increased *mdr1* expression can precede gene amplification. Science, **232**, 643-645.

Shen, D.W., Fojo, A.T., Roninson, I.B., Chin, J.E., Soffir, R., Pastan, I. & Gottesman, M.M. (1986C) Multidrug resistance of DNA-mediated transformants is linked to transfer of the human *mdr1* gene. Molecular and Cellular Biology, **6**, 4039-4045.

Sheppard, M.N., Corrin, B., Bennett, M., Marangos, P.J., Bloom, S.R. & Polak, J.M. (1984) Immunocytochemical localization of neurone specific enolase in small cell carcinomas and carcinoid tumours of the lung. Histopathology, **8**, 171-181.

Shiraishi, M., Morinaga, S., Noguchi, M., Shimosato, Y. & Sekiya, T. (1987) Loss of genes on the short arm of chromosome 11 in human lung carcinomas. Japanese Journal of Cancer Research, **78**, 1302-1308.

Siegfried, J.M., Tritton, T.R. & Sartorelli, A.C. (1983) Comparison of anthracycline concentrations in S180 cell lines of varying sensitivity. European Journal of Clinical Oncology, **19**, 1133-1141.

Sikora, K., Evan, G., Stewart, J. & Watson, J.V. (1985) Detection of the c-myc oncogene product in testicular cancer. British Journal of Cancer, **52**, 171-176.

Silvestrini, R., Daidone, M.G., Costa, A. & Sanfilippo, O. (1985) Cell kinetics and in vitro chemosensitivity as a tool for improved management of patients. European Journal of Cancer and Clinical Oncology, **21**, 371-378.

Simms, E., Gazdar, A.F., Abrams, P.G., & Minna, J.D. (1980) Growth of human small cell (oat cell) carcinoma of the lung in serum-free, growth factor - supplemented medium. Cancer Research, **40**, 4356-4363.

Simoons, M.L., Tooms, M., Lubsen, J. & Hugenholtz, P.G. (1980) Treatment of stable angina pectoris with verapamil hydrochloride - a double blind cross-over study. European Heart Journal, **1**, 269-274.

Singh, B.N., Ellrodt, G. & Peter, C.T. (1978) Verapamil: a review of its pharmacological properties and therapeutic use. Drugs, **15**, 169-197.

Sinha, B.K., Katki, A.G., Batist, G., Cowan, K.H. & Myers, C.E. (1987) Adriamycin-stimulated hydroxyl radical formation in human breast tumor cells. Biochemical Pharmacology, **36**, 793-796.

Sirotnak, F.M., Yang, C.H., Mines, L.S., Oribe, E. & Biedler, J.L. (1986) Markedly altered membrane transport and intracellular binding of vincristine in multidrug resistant Chinese hamster cells selected for resistance to vinca alkaloids. Journal of Cellular Physiology, **126**, 266-274.

Skipper, H.E. & Schabel, F.M., Jr. (1984) Tumor stem cell heterogeneity: implications with respect to classification of cancers by chemotherapeutic effect. Cancer Treatment Reports, **68**, 43-61.

Skovsgaard, T. (1978A) Mechanisms of resistance to daunorubicin in Ehrlich ascites tumor cells. Cancer Research, **38**, 1785-1791.

Skovsgaard, T. (1978B) Mechanisms of cross resistance between vincristine and daunorubicin in Ehrlich ascites tumor cells. Cancer Research, **38**, 4722-4727.

Skovsgaard, T., Dano, K. & Nissen, N.I. (1984) Chemosensitizers counteracting acquired resistance to anthracyclines and vinca alkaloids in vivo. A new treatment principle. Cancer Treatment Reviews, **11**, Suppl A, 63-72.

Sky-Peck, H.H. (1971) Effects of chemotherapy on the incorporation of ^3H -thymidine into DNA of human neoplastic tissue. National Cancer Institute Monograph, **34**, 197-203.

Slater, L.M., Murray, S.L., Watzel, M.W., Wisdom, R.M. & DuVall, E.M. (1982) Verapamil restoration of daunorubicin responsiveness in daunorubicin resistant Ehrlich ascites carcinoma. Journal of Clinical Investigation, **70**, 1131-1134.

Slater, L.M., Sweet, P., Stupecky, M. & Gupta, S. (1986A) Cyclosporin A reverses vincristine and daunorubicin resistance in acute lymphatic leukemia in vitro. Journal of Clinical Investigation, **77**, 1405-1408.

Slater, L.M., Sweet, P., Stupecky, M., Wetzels, M.W. & Gupta, S. (1986B) Cyclosporin A corrects daunorubicin resistance in Ehrlich ascites carcinoma. British Journal of Cancer, **54**, 235-238.

Slater, T.F., Sawyer, B. & Strauli, U. (1963) Studies on succinate-tetrazolium reductase systems: III Points of coupling of four different tetrazolium salts. Biochemica et Biophysica Acta, **77**, 383-393.

Slovak, M.L., Hoeltge, G.A., Dalton, W.S. & Trent, J.M. (1988) Pharmacological and biological evidence for differing mechanisms of doxorubicin resistance in two human tumor cell lines. Cancer Research, **48**, 2793-2797.

Smith, F.E. (1951) Tetrazolium Salt. Science, **113**, 751-754.

Sorenson, G.D., Pettengill, O.S., Brinck-Johnsen, T., Cate, C.C. & Maurer, L.H. (1981) Hormone production by cultures of small cell carcinoma of the lung. Cancer, **47**, 1289-1296.

Souhami, R.L., Bradbury, I., Geddes, D.M., Spiro, S.G., Harper, P.G. & Tobias, J.S. (1985) Prognostic significance of laboratory parameters measured at diagnosis in small cell carcinoma of the lung. Cancer Research, **45**, 2878-2882.

Sowerby, J.M. & Ottoway, J.H. (1962) Reduction of tetrazolium salts by phenazine methosulfate-linked oxidations. Biochemical Journal, **84**, 56-57.

Stephens, T.C., Adams, K. & Peacock, J.H. (1984) Identification of a sub-population of MeCCNU resistant cells in previously untreated Lewis lung tumors. British Journal of Cancer, **50**, 77-83.

Suda, T., Suda, J. & Ogawa, M. (1983) Proliferative kinetics and differentiation of murine blast cell colonies in culture: evidence for variable G₀ periods and constant doubling rates of early pluripotent hemopoietic progenitors. Journal of Cellular Physiology, **117**, 308-318.

Sugawara, I., Kataoka, I., Morishita, Y., Hamada, T., Tsuruo, T., Itoyama, S. & Mori, S. (1988) Tissue distribution of P-glycoprotein encoded by a multidrug-resistant gene as revealed by a monoclonal antibody, MRK 16. Cancer Research, **48**, 1926-1929.

Surewicz, W.K. & Jozwiak, Z. (1983) Effect of quinidine on membrane properties. Depression of the lipid phase transition temperature and changes in the permeability of the lipid bilayer. Biochemical Pharmacology, **32**, 1467-1471.

Sutherland, R.M., Eddy, H.A., Bareham, B., Reich, K. & Vanantwerp, D. (1979) Resistance to adriamycin in multicellular spheroids. International Journal of Radiation Oncology, Biology and Physics, **5**, 1225-1230.

Sutherland, R.M. & Durand, R.E. (1984) Growth and cellular characteristics of multicell spheroids. Recent Results in Cancer Research, **95**, 24-49.

Suzukake, K., Petro, B.J. & Vistica, D.T. (1982) Reduction in glutathione content of L-PAM resistant L1210 Cells confers drug sensitivity. Biochemical Pharmacology, **31**, 121-124.

Suzukake, K., Vistica, B.P. & Vistica, D.T. (1983) Dechlorination of L-phenylalanine mustard by sensitive and resistant tumor cells and its relationship to intracellular glutathione content. Biochemical Pharmacology, **32**, 165-167.

Tartaglione, T.A., Pieper, J.A., Lopez, L.L. & Mehta, J. (1983) Pharmacokinetics of verapamil and nor-verapamil during long-term oral therapy. Research Communications in Chemical Pathology and Pharmacology, **40**, 15-27.

Tew, K.D. & Clapper, M.L. (1988) Glutathione-S-transferases in anticancer drug resistance. In Mechanisms of Drug Resistance in Neoplastic Cells. ed. Wooley, P.V. & Tew, K.D. pp 141-159. New York: Academic Press.

Tew, K.D., Bomber, A.M. & Hoffman, S.J. (1988) Ethacrynic acid and piriprost as enhancers of cytotoxicity in drug resistant and sensitive cell lines. Cancer Research, **48**, 3622-3625.

Thiebaut, F., Tsuruo, T., Hamada, H., Gottesman, M.M., Pastan, I. & Willingham, M.C. (1987) Cellular localisation of the multidrug-resistance gene product P-glycoprotein in normal human tissues. Proceedings of the National Academy of Sciences of the United States of America, **84**, 7735-7738.

Tofilon, P.J., Wheeler, K.T. & Deen, D.F. (1984) Detection of heterogeneity in the chemosensitivity of 9L brain tumor cell lines to 1,3-Bis (2-chloroethyl)-1-nitrosourea by the sister chromatid exchange assay. European Journal of Cancer and Clinical Oncology, **20**, 927-931.

Triggle, D.J. & Swamy, V.C. (1983) Calcium antagonists. Some chemical-pharmacologic aspects. Circulation Research, **52**, 17-28.

Trope, C., Hakansson, L. & Dencker, H. (1975) Heterogeneity of human adenocarcinomas of the colon and the stomach as regards sensitivity to cytostatic drugs. Neoplasma, **22**, 423-429.

Tsukeda, H., Mizuno, S. & Nitta, K. (1978) Susceptibilities of normal and malignant human lung cells in culture to the cytocidal action of antitumor agents. Cancer Research, **38**, 2529-2532.

Tsuruo, T. & Fidler, I.J. (1981) Differences in drug sensitivity among tumor cells from parental tumors, selected variants and spontaneous metastases. Cancer Research, **41**, 3058-3064.

Tsuruo, T., Iida, H., Tsukagoshi, S. & Sakurai, Y. (1981) Overcoming of vincristine resistance in P388 leukemia in vivo and in vitro through enhanced cytotoxicity of vincristine and vinblastine by verapamil. Cancer Research, **41**, 1967-1972.

Tsuruo, T., Iida, H., Tsukagoshi, S. & Sakurai, Y. (1982) Increased accumulation of vincristine and adriamycin in drug-resistant P388 tumor cells following incubation with calcium antagonists and calmodulin inhibitors. Cancer Research, **42**, 4730-4733.

Tsuruo, T. (1983A) Reversal of acquired resistance to vinca alkaloids and anthracycline antibiotics. Cancer Treatment Reports, **67**, 889-894.

Tsuruo, T., Iida, H., Naganuma, K., Tsukagoshi, S. & Sakurai, Y. (1983B) Promotion by verapamil of vincristine responsiveness in tumor cell lines inherently resistant to the drug. Cancer Research, **43**, 808-813.

Tsuruo, T., Iida, H., Nojiri, M., Tsukagoshi, S. & Sakurai, Y. (1983C) Circumvention of vincristine and Adriamycin resistance in vitro and in vivo by calcium influx blockers. Cancer Research, **43**, 2905-2910.

Tsuruo, T., Iida, H., Tsukagoshi, S. & Sakurai, Y. (1983D) Potentiation of vincristine and Adriamycin effects on human haemopoietic tumor cell lines by calcium antagonists and calmodulin inhibitors. Cancer Research, **43**, 2267-2272.

Tsuruo, T., Iida, H., Kitatani, Y., Yokata, K., Tsukagoshi, S. & Sakurai, Y. (1984) Effects of quinidine and related compounds on cytotoxicity and cellular accumulation of vincristine and adriamycin in drug-resistant tumor cells. Cancer Research, **44**, 4303-4307.

Twentyman, P.R. (1980) Experimental chemotherapy studies: intercomparison of assays. British Journal of Cancer, **41**, 279-287.

Twentyman, P.R. (1985) Predictive chemosensitivity testing. British Journal of Cancer, **51**, 295-299.

Twentyman, P.R., Fox, N.E. & White, D.J. (1987) Cyclosporin A and its analogues as modifiers of adriamycin and vincristine resistance in a multi-drug resistant human lung cancer cell line. British Journal of Cancer, **56**, 55-57.

Twentyman, P.R. & Luscombe, M. (1987) A study of some variables in a tetrazolium dye (MTT) based assay for cell growth and chemosensitivity. British Journal of Cancer, **56**, 279-285.

Twentyman, P.R. (1988) Modification of cytotoxic drug resistance by non-immunosuppressive cyclosporins. British Journal of Cancer, **57**, 254-258.

Ueda, K., Cardarelli, C., Gottesman, M.M. & Pastan, I. (1987A) Expression of a full-length cDNA for the human "MDR1" gene confers resistance to colchicine, doxorubicin and vinblastine. Proceedings of the National Academy of Sciences of the United States of America, **84**, 3004-3008.

Ueda, K., Clark, D.P., Chen, C.J., Roninson, I.B., Gottesman, M.M. & Pastan, I. (1987B) The human multidrug resistance (mdr1) gene, cDNA cloning and transcription initiation. Journal of Biological Chemistry, **262**, 505-508.

Van der Bliek, A.M., Van der Velde-Koerts, T., Ling, V. & Borst, P. (1986) Overexpression and amplification of five genes in a multidrug resistant Chinese hamster ovary cell line. Molecular and Cellular Biology, **6**, 1671-1678.

Van der Bliek, A.M., Baas, F., Ten Houte de Lange, T., Kooiman, P.M., Van der Velde-Koerts, T. & Borst, P. (1987) The human mdr3 gene encodes a novel P-glycoprotein homologue and gives rise to alternatively spliced mRNAs in liver. EMBO Journal, **6**, 3325-3331.

Van Muijen, G.N., Ruiter, D.J., van Leeuwen, C., Prins, F.A., Rietsema, K. & Warnaar, S.O. (1984) Cytokeratin and neurofilament in lung carcinomas. American Journal of Pathology, **116**, 363-369.

Volm, M., Wayss, K., Kaufmann, M. & Mattern, J. (1979) Pretherapeutic detection of tumour resistance and the results of tumour chemotherapy. European Journal of Cancer, **15**, 983-993.

Von Hoff, D.D., Casper, J., Bradley, E., Sandback, J., Jones, D. & Makuch, R. (1981) Association between human tumor colony-forming assay results and response of an individual patient's tumor to chemotherapy. American Journal of Medicine, **70**, 1027-1041.

Walls, G.A. & Twentyman, P.R. (1985) Cloning of human lung cancer cells. British Journal of Cancer, **52**, 505-513.

Warr, J.R., Brewer, F., Anderson, M. & Fergusson, J. (1986) Verapamil hypersensitivity of vincristine resistant Chinese hamster ovary cell lines. Cell Biology International Reports, **10**, 389-399.

Waxman, H.L., Myerburg, R.J., Appel, R. & Sung, R.J. (1981) Verapamil for control of ventricular rate in paroxysmal supraventricular tachycardia and atrial fibrillation or flutter- a double-blind, randomized cross-over study. Annals of Internal Medicine, **94**, 1-6.

Weisenthal, L.M., Dill, P.L., Kurnick, N.B. & Lippman, M.E. (1983A) Comparison of dye exclusion assays with a clonogenic assay in the determination of drug-induced cytotoxicity. Cancer Research, **43**, 258-264.

Weisenthal, L.M., Marsden, J.A., Dill, P.L. & Macaluso, C.K. (1983B) A novel dye exclusion method for testing in vitro chemosensitivity of human tumors. Cancer Research, **43**, 749-757.

Weisenthal, L.M., Shoemaker, R.H., Marsden, J.A., Dill, P.L., Baker, J.A. & Moran, E.M. (1984) In vitro chemosensitivity assay based on the concept of total tumor cell kill. Recent Results in Cancer Research, **94**, 161-173.

Weisenthal, L.M. & Lippman, M.E. (1985) Clonogenic and nonclonogenic in vitro chemosensitivity assays. Cancer Treatment Reports, **69**, 615-632.

West, G.W., Weichselbaum, R. & Little, J.B. (1980) Limited penetration of methotrexate into human osteosarcoma spheroids as a proposed model for solid tumour resistance to adjuvant chemotherapy. Cancer Research, **40**, 3665-3668.

Whang-Peng, J., Kao-Shan, C.S., Lee, E.C., Bunn, P.A., Carney, D.N., Gazdar, A.F. & Minna, J.D. (1982) Specific chromosome defect associated with human small-cell lung cancer; deletion 3p (14-23) Science, **215**, 181-182.

Wheeler, C., Rader, R. & Kessel, D. (1982) Membrane alterations associated with progressive adriamycin resistance. Biochemical Pharmacology, **31**, 2691-2693.

Wheeler, H., Bell, D. & Levi, J. (1988) A randomized study of doxorubicin, etoposide and vindesine with or without verapamil, in small cell lung cancer (SCLC). Proceedings of the American Society of Clinical Oncology, **7**, 208 (abstract).

Wheldon, T.E., Berry, I., O'Donoghue, J.A., Gregor, A., Hann, I.M., Livingstone, A., Russel, J. & Wilson, L. (1987) The effect on human neuroblastoma spheroids of fractional radiation regimes calculated to be equivalent for damage to late responding normal tissues. European Journal of Cancer and Clinical Oncology, **23**, 855-860.

Willingham, M.C., Cornwell, M.M., Cardarelli, C.O., Gottesman, M.M. & Pastan, I. (1986) Single cell analysis of daunomycin uptake and efflux in multidrug-resistant and sensitive KB cells: effects of verapamil and other drugs. Cancer Research, **46**, 5941-5946.

Wolberg, W.H. (1972) Response of DNA thymine synthesis in human tumor and normal tissue to 5-fluorouracil. Cancer Research, **32**, 130-132.

Wurster-Hill, D.H., Cannizzaro, L.A., Pettengill, O.S., Sorsenson, G.D., Cate, C.C. & Maurer, L.H. (1984) Cytogenetics of small cell carcinoma of the lung, Cancer Genetics and Cytogenetics, **13**, 303-330.

Yalowich, J.C., Zucali, J.R., Gross, M. & Ross, W.E. (1985) Effects of verapamil on etoposide, vincristine, and adriamycin activity in normal human bone marrow granulocyte-macrophage progenitors and in human K562 leukemia cells in vitro. Cancer Research, **45**, 4921-4924.

Yesner, R. & Carter, D. (1982) Pathology of carcinoma of the lung. Changing patterns. Clinics in Chest Medicine, **3**, 257-289.

Yuhas, J.M., Tarleton, A.E. & Harmon, J.G. (1978) In vitro analysis of the response of multicellular tumor spheroids exposed to chemotherapeutic agents in vitro or in vivo. Cancer Research, **38**, 3595-3598.

Yunis, J.J. (1983) A chromosomal basis of human neoplasia. Science, **221**, 227-236.

